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Selective extraction of polysaccharide affects the adsorption of proanthocyanidin by grape cell walls

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ABSTRACT

Cell wall material was isolated from two *Vitis vinifera* grape varieties, Cabernet Sauvignon and Shiraz, following a buffered phenol extraction method. Using sequential fractionation in chelating agent, then increasing the molarity of aqueous potassium hydroxide, polysaccharide classes were selectively extracted from cell walls to produce fractions of defined polysaccharide composition. Following the application of phloroglucinolysis and a modified HCl-butanol colourimetric assay to cell wall fractions, more than 54% of cell wall-bound proanthocyanidin was localised within the chelator-soluble (pectic) fraction. Model adsorption experiments with a purified skin proanthocyanidin confirmed that the removal of pectic polysaccharides by chelator most significantly reduced the adsorption of proanthocyanidin by cell walls. Nevertheless, cell wall hemicellulosic fractions retained a high binding capacity for proanthocyanidin, although lower than that observed when pectin was present. Following removal of hemicelluloses by fractionation, the primarily lignocellulosic residue had a significantly reduced affinity for proanthocyanidin. With the exception of lignocellulose, a greater selectivity of adsorption for higher molecular mass proanthocyanidins was observed by the respective cell wall fractions.

1. Introduction

The specific adsorption of condensed tannin (proanthocyanidin) by cell wall polysaccharides has been reported for various plant species (Bindon, Smith, & Kennedy, 2010; Perez-Jimenez, & Torres, 2011; Renard, Baron, Guyot, & Drilleau, 2001). In proanthocyanidin-rich foods, this phenomenon has been recognised as important in facilitating the retention, extraction or conversion of proanthocyanidin during processing (Bindon, Smith, Holt, & Kennedy, 2010; Le Bourvellec, Watrelot, Ginies, Imberty, & Renard, 2012; Le Bourvellec, Le Quere, & Renard, 2007). The presence of a significant quantity of proanthocyanidin in plant cell wall residues limits the extractability of polysaccharides, as well as the accessibility of polysaccharide-cleaving enzymes, in particular the highly methylated pectic fraction (Le Bourvellec, Guyot, & Renard, 2009). In light of this, the propensity of proanthocyanidins to bind polysaccharide appears to be conferred, in part, by the composition of the polysaccharide. Studies using model polysaccharides have shown that proanthocyanidin preferentially binds pectin to a greater extent than xyloglucan, which in turn has a greater affinity than cellulose (Le Bourvellec, Bouchet, & Renard, 2005). This hierarchy of polysaccharide-proanthocyanidin affinity is partly conferred by the three dimensional structure either limiting or facilitating access of proanthocyanidin molecules to polysaccharide binding sites. Compositionally, the presence of methylated galacturonans within pectic polysaccharides is noted to enhance proanthocyanidin binding (Le Bourvellec et al., 2009; Watrelot, Le Bourvellec, Imberty, & Renard, 2013). A study has shown that the selective removal of the pectic fraction of apple cell walls by boiling decreases the affinity of cell wall material for proanthocyanidin, and a strong interaction of the solubilised pectin and proanthocyanidin was confirmed *via* isothermal titration calorimetry (Le Bourvellec et al., 2012). Nevertheless, a relatively high residual affinity of cell wall material for proanthocyanidin was retained, even following partial removal of pectic polysaccharides (Le Bourvellec et al., 2012). It was noteworthy that altering the protein content of cell walls was found not to affect the adsorption of proanthocyanidin (Le Bourvellec et al., 2012). These observations highlight the significant role that polysaccharides play in mediating cell wall–proanthocyanidin interactions. Given the diversity of polysaccharide structures that exist within cell walls, a knowledge gap can be identified in terms of the compositional characteristics which confer polysaccharide affinity for proanthocyanidin.

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An increasing body of evidence shows that the composition of proanthocyanidin is also important in determining binding events with cell walls, in addition to polysaccharide composition (Bindon & Kennedy, 2011; Bindon, Smith, Holt, et al., 2010; Bindon, Smith, & Kennedy, 2010; Renard et al., 2001). In general, larger proanthocyanidins are selectively adsorbed by cell walls (Le Bourvellec, Guyot, & Renard, 2004; Renard et al., 2001), and this is found to be somewhat independent of molecular mass, where proanthocyanidins occupying a greater hydrodynamic volume are more effectively bound (Bindon, Smith, Holt, et al., 2010). An exception has been found for very high molecular mass proanthocyanidins (>15,000 g/mol) from the skins of red grapes, which appear to be excluded from binding events when cell wall porosity is low (Bindon, Madani, Pendleton, Smith, & Kennedy, 2014; Bindon, Bacic, & Kennedy, 2012; Bindon & Kennedy, 2011; Bindon, Smith, & Kennedy, 2010). Increasing the porosity of the cell wall may enhance the adsorption of high molecular mass proanthocyanidin, which may in turn reduce their extractability. In light of this, an understanding of proanthocyanidin-cell wall interactions in grapes is important, since this may limit proanthocyanidin extraction during vinification (Bindon, Smith, & Kennedy, 2010; Hanlin, Hrmova, Harbertson, & Downey, 2010; Pinelo, Arnous, & Meyer, 2006).

Winemaking techniques can potentially make a significant impact on the interaction of proanthocyanidin and cell walls. In red wine ferments conducted with skin contact, the progression of fermentation results in the solubilisation of polysaccharides rich in galacturonic acid, arabinose and galactose (Guadalupe & Ayestaran, 2007). It has been found that the application of flash treatment can enhance the extraction of arabinose- and galactose-enriched polysaccharides, as well as rhamnogalacturonan II, but only when fermentation following the flash treatment is conducted with skin contact (Doco, Williams, & Cheynier, 2007). Since both the fermentation and flash release processes result in the co-solubilisation of tannins and skin-derived polysaccharides (Morel-Salmi, Souquet, Bes, & Cheynier, 2006), the potential exists that the processes of polysaccharide and tannin release from grape solids are related. Conceptually, the winemaking process may limit the adsorption of tannins by grape cell walls by removing polysaccharides of a high tannin binding capacity, facilitating their co-solubilisation, or simply by altering the conditions of the tannin–cell wall interaction (e.g. heat or ethanol during fermentation) (Pinelo et al., 2006; Cholet et al., 2014).

To explain the effect of the winemaking process on tannin–cell wall interactions in grape skins and provide clues as to how this can be manipulated, an understanding of the contribution of polysaccharide classes in conferring cell wall binding capacity for tannin is required. To our knowledge, only limited published work exists (Nunan, Sims, Bacic, Robinson, & Fincher, 1998; Moore et al., 2014) which describes the compositional investigation of the cell walls of grapevine using fractionation techniques, and none for grape skins. Furthermore, while some evidence exists which indicates that proanthocyanidin is present as a bound fraction within grape cell walls (Bindon et al., 2012; Bindon, Smith, & Kennedy, 2010; Downey, Harvey, & Robinson, 2003; Verries et al., 2008; Cholet et al., 2014), the localisation and composition of proanthocyanidin within defined cell wall fractions is unknown.

The current study has sought to characterise and quantify polysaccharide classes within the cell walls of ripe grapes of two important commercial cultivars using sequential fractionation in a chelating agent, followed by increasing concentrations of KOH. Using purified grape skin proanthocyanidin, the adsorption properties of each cell wall fraction for proanthocyanidin was determined. To further confirm the presence of residual proanthocyanidin in cell wall fractions, acid-catalysed depolymerisation techniques were used. The work shows that polysaccharide composition is important in defining the adsorption response of cell walls for proanthocyanidin. The commercial implications of these findings are discussed.

2. Materials and methods

2.1. Instrumentation

An Agilent model 1100 HPLC (Agilent Technologies Australia Pty Ltd, Melbourne, Victoria, Australia) was used with Chemstation software for chromatographic analyses.

2.2. Grape sample collection and storage

Vitis vinifera L. cv. Cabernet Sauvignon and Shiraz grape samples were obtained from single, neighbouring blocks in a commercial vineyard (Pernod Ricard Australia, Orlando Wines) in the Langhorne Creek growing region of South Australia (350° 16′ 11.56″S, 1390° 00′ 14.47″E, elevation 28 m). The respective Cabernet Sauvignon and Shiraz blocks had grapevines of the same age (13 years), and both were on own roots. The planting density was 2.5 m between rows × 1.8 m between grapevines. For each grape variety, harvests were on 13th March and 16th April, corresponding to juice soluble solids levels of 23 °Brix and 25 °Brix, respectively. Whole-bunch samples (36) were collected from three rows distributed within the vineyard block to obtain a representative sample and were frozen whole at −80 °C until further analysis. Triplicate 50-berry samples were removed from frozen bunches, weighed, and skins were then separated from flesh and seed, while kept on dry ice. Frozen skin material was stored in 50 mL screw-cap centrifuge tubes at −80 °C until further processed.

2.3. Extraction and purification of grape proanthocyanidin

The 50 berry samples yielded between 10 g and 12 g of fresh grape skins. Skins were extracted for 18 h in 35 mL 70% v/v aqueous acetone containing 0.01% v/v trifluoroacetic acid (TFA). Extractions were performed on a Ratek suspension mixer (Ratek Instruments Pty Ltd., Boronia, Victoria, Australia) for 18 h, in the dark. Following extraction, whole skin fragments were recovered by filtering through a 0.5 mm mesh. Extracts were retained and centrifuged at 8000 × *g* for 5 min. The recovered skin material was re-suspended in 35 mL of fresh 70% v/v acetone 0.01% v/v TFA and homogenised using an Ultra-Turrax T25 high-speed homogeniser with a S25N dispersing head (Janke & Kunkel GmbH & Co., Germany) at 24,000 rpm for 2 min. The homogenate was then further extracted for 18 h, with agitation on a suspension mixer. Extracts were then centrifuged at 8000 × *g* for 5 min, and the supernatant recovered. The 70% acetone-insoluble skin material was then frozen at −80 °C in a pre-tared centrifuge tube, dried under vacuum at ≤ −50 °C, and the recovered dry weight recorded. A 25 mL aliquot of the 70% v/v acetone extracts was concentrated under reduced pressure at 35 °C, and reconstituted in 30 mL of 60% (v/v) HPLC grade methanol containing 0.05% v/v TFA. Reconstituted extracts were then purified by affinity chromatography on Sephadex LH20 chromatography resin (Amersham, Uppsala, Sweden) according to the conditions described previously (Bindon & Kennedy, 2011) and quantitatively recovered as dry powders following lyophilisation. Dry skin proanthocyanidin powders were stored at −20 °C under nitrogen.

2.4. Preparation of grape skin cell walls

Cell wall material (CWM) was prepared from dry 70% acetone grape skin residues using the method of Vidal, Williams, O'Neill, and Pellerin (2001) with the following modifications. A 50 mg sample of

dry 70% acetone-extracted skin material was retained, and stored at −20 °C. The remaining skin material was extracted by slow shaking for 30 min in 25 mL Tris–HCl equilibrated phenol pH 6.7 (Sigma-Aldrich, St. Louis, MO, USA), and the phenol-insoluble CWM residue recovered following filtration on glass microfiber. The CWM was then washed twice with 50 mL 80% v/v ethanol, and 50 mL acetone to remove phenol. Samples were then extracted with slow shaking for 30 min in 25 mL 1:1 v:v methanol: chloroform, and the CWM was washed twice with 50 mL methanol and twice with 50 mL acetone. CWM was recovered by filtration on glass microfiber, and solvent removed under vacuum at ≤−50 °C. CWM was manually ground to a fine particle size with a mortar and pestle, passed through a 0.5 mm mesh, and frozen at −20 °C until used.

2.5. Fractionation of grape skin cell walls

The fractions of the CWM were obtained using an adaptation of established methods (Selvendran, 1985; Selvendran & O'Neill, 1987) with modifications as follows. The process was divided into four fractionation steps (Fig. 1). For the first extraction step (F1), 10 mg of sample was weighed into a pre-tared 1.5 mL screw-cap centrifuge tube and extracted overnight with 1 mL of 50 mM *trans*-1,2-diaminocyclohexane *N,N,N′,N′*-tetracetic acid monohydrate (CDTA) pH 6.5 (Sigma-Aldrich, St. Louis, MO, USA), shaking at 17 °C. Thereafter, samples were centrifuged at 21,000 × *g* for 10 min. The supernatant was recovered, and an 800 μL aliquot was dialysed using molecular weight cut-off 3500 Da using 1 mL Pur-a-Lyzer dialysis tubes (Sigma-Aldrich, St. Louis, MO, USA), first against 1 M NaCl and then against 3 changes of Milli-Q water. After dialysis, each sample was transferred to a fresh screw-cap centrifuge tube, and the dialysis tube washed with an additional 800 μL of Milli-Q water to ensure that any insoluble polysaccharide residue was recovered. Thereafter, the dialysed samples were frozen at −80 °C and lyophilised at ≤−50 °C (F1 extract). The residue (F1 CWM) was washed 3 times with Milli-Q water, frozen at −80 °C, and lyophilised at ≤−50 °C. The gravimetric recovery of the dry residue was recorded. The remaining extraction steps employed increasing concentrations of KOH, each containing 20 mM $NaBH_4$. The extraction solvents were F2, 50 mM KOH; F3, 1 M KOH and F4, 4 M KOH. For the production of extracts and residues of F2, F3 and F4 (Fig. 1), the extraction was performed sequentially on the residue of the CDTA extraction, using 1 mL of the respective extraction solutions. Extractions were conducted over 2 h, with continuous shaking, at 17 °C, followed by centrifugation at 21,000 × *g* for 10 min. The extract supernatants were recovered, neutralised with glacial acetic acid, and dialysed using molecular weight cut-off 3500 Da against 3 changes of Milli-Q water. Dialysed extracts were frozen at −80 °C, lyophilised at ≤−50 °C, and designated F2 extract, F3 extract and F4 extract depending upon the extraction sequence followed (Fig. 1). Between each extraction step, the residue was further washed twice with 1 mL Milli-Q water, centrifuged at 21,000 × *g* for 10 min and the wash supernatant removed prior to application of the following extraction solvent. After the final extraction was completed, the samples were centrifuged, the CWM residue was washed 3 times with Milli-Q water, frozen at −80 °C and lyophilised at ≤−50 °C. The gravimetric recovery of the dry CWM residues of F2, F3 and F4 were recorded. All extractions were performed in triplicate.

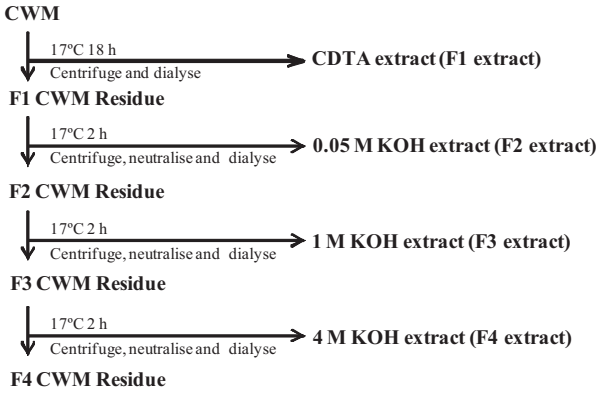

Fig. 1. Summary of the cell wall (CWM) fractionation procedure indicating points at which extracts and residue were recovered for analysis.

2.6. Proanthocyanidin compositional analysis

Proanthocyanidin was characterised by phloroglucinolysis (Kennedy & Jones, 2001; Kennedy & Taylor, 2003) to determine subunit yield, composition and mean degree of polymerisation (mDP). Phloroglucinolysis was performed on proanthocyanidin isolates reconstituted in methanol, or directly on whole CWM and CWM extraction residues according to the conditions described previously (Bindon, Smith, Holt, et al., 2010; Bindon, Smith, & Kennedy, 2010). The phloroglucinolysis reaction was carried out at 50 °C for 25 min, neutralised and analysed by RP-HPLC according to the conditions outlined in (Kennedy & Taylor, 2003) using (−)-epicatechin (Sigma Aldrich, St. Louis, MO, USA) as the quantitative standard. To a 25 μL aliquot of selected methanolic skin proanthocyanidin samples, 100 μL of *N,N*-dimethylformamide (DMF) was added. These samples were directly analysed by gel permeation chromatography (GPC), according to the method of Kennedy and Taylor (2003) with the modifications previously described (Bindon & Kennedy, 2011). Preveraison skin proanthocyanidin fractions of known molecular mass (by phloroglucinolysis) were used as standards for GPC calibration. For calibration, a second order polynomial was fitted with the proanthocyanidin elution time at 50% for each standard.

2.7. Proanthocyanidin quantification

Using phloroglucinolysis directly on cell wall fractions, as described above, proanthocyanidin could not be detected in any sample following extraction in KOH. Therefore, an additional analysis of proanthocyanidin in CWM and CWM fractions was performed by the method previously described by Grabber, Zeller, and Mueller-Harvey (2013). Briefly, butanol–HCl reagent was prepared freshly every day by stirring 42 mg of ammonium iron (III) sulfate dodecahydrate in 5.2 mL of 12 M HCl and then adding 46 mL of acetone. The solution was made up to 100 mL with butanol. A 2.85 mL aliquot of reagent was added to 10 mg of the CWM suspended in 150 μL of 70% acetone in sealed glass tubes. The tubes were heated at 70 °C in a water bath, with continuous shaking for 2.5 h. Samples were cooled on ice, and centrifuged. The absorbance at 550 nm was measured on a dual beam Varian Cary 300 Scan UV–visible spectrophotometer (Varian, Melbourne, Australia). As a quantitative standard, a preveraison (green, unripe) grape skin tannin standard of a high conversion yield by phloroglucinolysis (>70%) was used which was purified as described previously (Kennedy & Jones, 2001). In order to compare the proanthocyanidin concentration in CWM fractions and whole-skin acetone extracts, this standard was analysed using a high-throughput adaptation of the methyl cellulose precipitable tannin (MCPT) assay (Mercurio, Dambergs, Herderich, & Smith, 2007) to prepare an external standard curve. Five mL of the 70% v/v acetone extracts prepared from the extraction of whole grape skins were dried under a stream of nitrogen, recovered in the same volume of 15% v/v aqueous ethanol containing 0.01% v/v TFA, and analysed using MCPT, and quantitatively expressed as proanthocyanidin units (mg) using the external standard curve.

2.8. Effect of potassium hydroxide on proanthocyanidin colour development in butanol–HCl

It was expected that the extraction of the CWM with KOH could result in oxidation of the cell wall bound proanthocyanidin (Cilliers & Singleton, 1990) potentially reducing coloured anthocyanidin yield in the butanol-HCL assay. The effect of alkaline conditions on proanthocyanidin was tested experimentally using 10 mg of purified skin proanthocyanidin to which 250 μL of 4 M KOH, 20 mM $NaBH_4$ was added. The sample was vortexed, sealed and left at room temperature for 30 min, and thereafter adjusted to pH 6 with glacial acetic acid. To a control proanthocyanidin sample, 4 M KOH/acetic acid solution, pH 6, was added. The alkaline-treated and control samples were diluted to 1 mg/mL with 70% v/v acetone, and subjected to analysis as described above. It was found that the KOH step significantly decreased the development of colour at 550 nm, yielding 42% of the control. The analysis of CWM fractions F2 to F4 was performed as described above, and it was assumed that a colour yield at 550 nm indicated the presence of proanthocyanidin in the material. However, due to the potential reduction in colour development in the assay after exposure to KOH, this was not assumed to present a quantitative assessment.

2.9. Binding reaction of proanthocyanidin and cell wall fractions

An experiment was designed to determine the sequential loss of cell wall binding capacity for proanthocyanidin following sequential fractionation of CWM. For this experiment 10 mg of CWM was weighed into 1.5 mL centrifuge tubes, or extraction residues (F1–F4 CWM residue) isolated from 10 mg of CWM starting material were retained in the same 1.5 mL centrifuge tube used for fractionation. CWM fractions were combined with a 1 mL aliquot of a 2 mg/mL sample of purified grape proanthocyanidin of a high mass conversion (62% w/w) (Bindon & Kennedy, 2011) in 12% v/v aqueous ethanol containing 0.01% TFA. Cell wall-proanthocyanidin solutions were vortexed, and then incubated for 1 h at 27 °C with shaking, and thereafter centrifuged at 21,000 × *g* for 10 min. For each experiment a standard blank of the respective proanthocyanidin solution without cell wall material was included. An aliquot of the supernatant was concentrated under vacuum at 30 °C in a Heto vacuum centrifuge (Heto-Holten A/S, Allerod, Denmark), recovered in methanol, and analysed by phloroglucinolysis and GPC as described above. The loss of proanthocyanidin in supernatants through interaction with CWM was determined by the difference in phloroglucinolysis yield from the control. Experiments were performed in triplicate on biological replicates of grape cell wall preparations.

2.10. Monosaccharide composition of cell wall fractions

Lyophilised cell wall extracts of F1 to F4 (F1–F4 extract, Fig. 1) were hydrolysed in 2 M TFA at 100 °C for 3 h. Hydrolysates were cooled on ice, and thereafter concentrated under vacuum at 30 °C in a Heto vacuum centrifuge (Heto-Holten A/S, Allerod, Denmark). For un-fractionated CWM and F4 CWM, samples were hydrolysed in 12 M H_2SO_4 for 3 h at room temperature, then diluted to 1 M H_2SO_4 with Milli-Q water and placed at 100 °C for a further 3 h. Samples were cooled on ice, and neutralised with 4 M NaOH. Samples were centrifuged at 21,000 × *g* for 10 min, and the supernatant removed for monosaccharide analysis. The hydrolysis residue, hereafter referred to as Klason lignin, was washed 3 times with Milli-Q water, lyophilised, and the dry weight recorded.

For the analysis of hydrolytically-released monosaccharides an adaptation of the method of Honda, Akao, Suzuki, Okuda, Kakehi, and Nakamura (1989) was used. The dry TFA-hydrolysates were resuspended in Milli-Q water containing 0.3 M of ribose and deoxyglucose as internal standards (Sigma-Aldrich, St. Louis, MO, USA). For H_2SO_4 hydrolysates, no concentration step was carried out, and neutralised samples were diluted 1:1 with 0.6 M internal standard in Milli-Q water. The derivatising reagent was 0.5 M of methanolic 1-phenyl-3-methyl-5-pyrazolone (PMP) in 1 M NH_4OH. For the derivatisation, 25 μL of sample was mixed with 96.2 μL of derivatising reagent and placed in a heating block at 70 °C for 1 h. After this step, the samples were cooled on ice and neutralised with 25 μL of 10 M formic acid. Then, the samples were extracted twice with dibutyl ether (Sigma-Aldrich, St. Louis, MO, USA) and the upper layer was discarded. The excess of dibutyl ether was dried under vacuum at room temperature. PMP-monosaccharide derivatives were quantified by HPLC using a C18 column (Kinetex, 2.6 μm, 100 Å, 100 × 3.0 mm) protected with a guard cartridge (KrudKatcher Ultra HPLC in-line filter, 0.5 μm) (Phenomenex, Lane Cove, NSW, Australia). The mobile phases were solvent A, 10% (v/v) acetonitrile in 40 mM aqueous ammonium acetate, and solvent B, 70% (v/v) acetonitrile in water. The following linear gradient was used: for solvent A (with solvent B making up the remainder) 92% at 0 min; 84% at 12 min; to 0% at 12.5 min; 0% at 14 min, then returning to the starting conditions at 14.5–18.5 min, 92%. A flow rate of 0.6 mL/min was used with a column temperature of 30 °C. The PMP-monosaccharide derivatives were identified using commercial standards (Sigma-Aldrich, St. Louis, MO, USA). The recovery of monosaccharides did not differ significantly between whole CWM samples, and was ±33% by weight following hydrolysis in H_2SO_4 which is in agreement with published work on grape skin cell walls using the protocol described (Bindon et al., 2012; Bindon & Smith, 2013). The sum of recovered monosaccharides for the extracts F1 to F4 and F4 residue was approximately 90% of total monosaccharides for CWM determined following H_2SO_4 hydrolysis, indicating acceptable recoveries.

2.11. Degree of acetylation and methylation of galacturonans

To 30 mg of whole unfractionated CWM, 2 mL 0.4 N NaOH in 1:1 v/v isopropanol:water was added. Samples were vortexed and placed in an ice bath for 1 h, and then maintained or 2 h at room temperature with intermittent vortexing (3 times). Samples were then centrifuged for 5 min. The methanol and acetic acid content of supernatants was determined by HPLC according to the method described by Voragen, Schols, and, Pilnik (1986). An ORH-801 column (300 mm × 6.5 mm, 5 μm particle size) equipped with an ORH-801 guard column was used (Interaction, San Jose, CA, USA). The eluent was 5 mM sulphuric acid at a constant flow of 0.8 mL/min with a column temperature of 30 °C, with refractive index detection. Pure methanol and glacial acetic acid (Panreac, Barcelona, Spain) were used as analytical standards. The degree of methylation and the degree of acetylation was calculated as the molar ratio of NaOH-released methanol and acetic acid to anhydrogalacturonic acid × 100, using the concentration of galacturonic acid as mg/g CWM following the hydrolysis in sulphuric acid as described above.

2.12. Statistical analysis

Data were analysed by one-way or two-way analysis of variance (ANOVA) using the JMP 5.0.1 statistical software package (SAS, Cary, NC, USA). ANOVAs were followed by a post hoc Tukey's HSD test to determine differences between means.

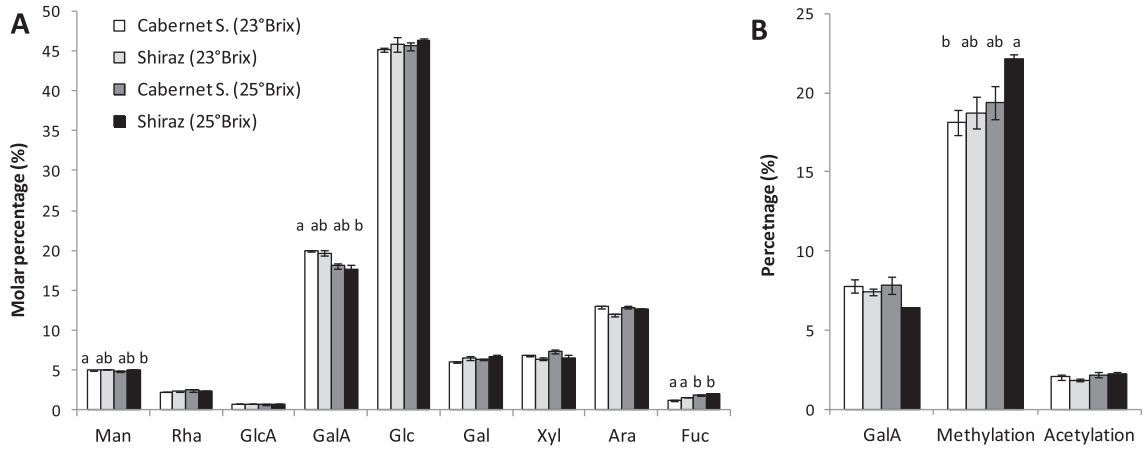

Fig. 2. Analysis of whole cell wall materials from two grape varieties and ripeness stages for (A) monosaccharide composition as molar proportion following harsh acid hydrolysis (sulphuric) and (B) degree of methylation and acetylation of galacturonic acid. Data were compared by one-way ANOVA, $n = 12$, followed by a Tukey's post-hoc test where different letters indicate significant differences between means. Abbreviations: Man—mannose, Rha—rhamnose, Glc A—glucuronic acid, Gal A—galacturonic acid, Glu—glucose, Gal = galactose, Xyl—xylose, Ara—arabinose, Fuc—fucose.

3. Results and discussion

3.1. Gravimetric recovery and monosaccharide yield in cell wall fractions

The recovered yield of cell wall material (F0) on a per berry basis was similar for the two grape varieties and ripeness stages for which samples were analysed (Table 1). In comparing the composition of the whole CWMs following harsh acid hydrolysis, minor differences were found between the samples in terms of galacturonic acid, mannose and fucose (Fig. 2A). Lowered galacturonic acid by molar percentage of total monosaccharides was in turn reflected by an increase in the degree of galacturonan methylation (Fig. 2B). However, this was independent of changes in the concentration of galacturonic acid, which did not change significantly between samples.

Following sequential fractionation of the cell walls (F0) as 10 mg batches, a distinct series of residues were obtained, which were recovered gravimetrically (Table 1). The greatest loss of material following fractionation was following CDTA extraction (F1, 24% loss) and 50 mM KOH (F2, 26% loss). Extraction in increasing molarity of 1 M and 4 M KOH gave losses of 11% (F3) and 9% (F4), respectively. A further significant loss in material occurred following harsh acid hydrolysis of the F4 residue (21%), yielding approximately 10% by mass of Klason lignin. The yield of lignin was similar to that previously observed for grape skin CWM following sequential fractionation in detergent, followed by acid-hydrolysis (Bindon, Smith, & Kennedy, 2010) but was far lower than that determined using the traditional Klason lignin procedure, as we have noted previously in these type of samples (Bindon et al., 2012). It is therefore likely that a considerable portion of lignin was co-extracted with hemicelluloses during fractionation with KOH.

The polysaccharide content of each fraction, determined by acid-hydrolysis and monosaccharide analysis of extracts is shown in Tables 2 and 3. The proportion of monosaccharide recovered after each fractionation step was poorly related to the dry weight removed. The lowest conversion to monosaccharides by weight was from extracts of F1 and F2, intermediate for extracts of F3 and F4, and highest following acid-hydrolysis of the F4 residue. Due to the selection of 2 M TFA for the hydrolysis of extracts of F1 to F4, it could be expected that low yield of monosaccharides may reflect a less effective hydrolysis using TFA by comparison with sulphuric acid. However, based on the similarities of monosaccharide recovery from each fraction and total yield following harsh acid-hydrolysis of whole CWM (F0), it is unlikely that the effect was due to the hydrolysis method itself. It is more likely to be due to incomplete hydrolysis of galacturonans, or degradation of released galacturonic acid subunits concomitant with the hydrolysis procedure (Li, Kisara, Danielsson, Lindstrom, & Gellerstedt, 2007). The use of TFA hydrolysis has also been reported to yield lower levels of monosaccharides from neutral polysaccharides than harsh acid hydrolysis (Arnous & Meyer, 2009). Alternatively, the presence of significant quantities

Table 1
Gravimetric recoveries of whole cell wall material (F0) and cell wall fractions (F1 to F4, and lignin) from two grape varieties and two ripeness stages, showing their assigned polysaccharide classes based on monosaccharide analysis.

Fraction	Extraction Method‡	Component Extracted‖	Unit	Cabernet S. (23 °Brix)	Gravimetric recovery of residue†		
					Cabernet S. (25 °Brix)	Shiraz (23 °Brix)	Shiraz (25 °Brix)
F0	Buffered phenol	CWM	mg per berry	9.77 ± 0.87	10.17 ± 0.83	10.92 ± 1.11	11.16 ± 1.06
F1	0.05 M CDTA	Pectin, AG Type 1, Arabinan	mg per 10 mg F0	7.62 ± 0.11	7.57 ± 0.11	7.75 ± 0.05	7.66 ± 0.14
F2	0.05 M KOH	Arabinan, AG Type 1, Pectin, XG		5.15 ± 0.11	5.18 ± 0.17	5.00 ± 0.17	5.06 ± 0.04
F3	1 M KOH	GAX, HM, XG		3.90 ± 0.10	3.97 ± 0.17	4.13 ± 0.13	3.97 ± 0.03
F4	4 M KOH	XG, HM		3.13 ± 0.09	3.13 ± 0.12	3.47 ± 0.22	2.83 ± 0.03
Lignin	Acid hydrolysis	Cellulose		0.96 ± 0.08	1.00 ± 0.10	1.20 ± 0.05	0.97 ± 0.08

† Total cell wall material (F0) prepared from a 50 berry sample expressed as mg per berry, thereafter, gravimetric recovery of fractions F1 to F4 and lignin expressed as mg recovered per 10 mg of F0.
‡ CDTA—*trans*-1,2-diaminocyclohexane *N*,*N*,*N*′,*N*′-tetracetic acid monohydrate, acid hydrolysis—12 M H_2SO_4 for 3 h at 25 °C diluted to 1 M, then 100 °C for 3 h.
‖ CWM—cell wall material, AG—arabinogalactan, GAX—glucuronoarabinoxylan, HM—heteromannan, XG—xyloglucan; data are mean ± standard error, $n = 3$.

Table 2
Monosaccharide recovery and composition for cell wall fractions of Cabernet Sauvignon grape skins at two ripeness stages.

	Total yield	Monosaccharide composition (mole %)[†]								
	(mg/g F0)	Man	Rha	GlcA	GalA	Glc	Gal	Xyl	Ara	Fuc
23 °Brix										
F1 extract[‡]	24.1[b]	1.62[e]	4.25[b]	2.96[a]	43.16[b]	1.61[e]	17.72[a]	3.69[c]	23.44[c]	1.55[c]
F2 extract	25.1[b]	0.77[e]	8.82[a]	2.97[a]	16.37[c]	3.98[de]	13.85[b]	4.04[c]	47.90[a]	1.30[c]
F3 extract	45.0[b]	5.81[c]	4.51[b]	1.29[b]	3.08[e]	14.93[c]	9.81[c]	29.44[a]	28.55[b]	2.58[b]
F4 extract	28.4[b]	16.56[a]	1.10[d]	0.88[c]	0.39[f]	35.12[b]	10.31[c]	26.91[b]	4.19[e]	4.54[a]
F4 residue	171.9[a]	4.66[cd]	1.11[d]	0.23[d]	2.31[e]	81.22[a]	2.27[d]	0.56[d]	7.18[d]	0.44[d]
25 °Brix										
F1 extract	25.2[b]	1.06[e]	4.07[b]	2.65[a]	44.40[a]	1.75[e]	16.78[a]	2.89[c]	23.60[c]	2.79[b]
F2 extract	27.3[b]	0.70[e]	8.52[a]	2.85[a]	14.31[d]	5.28[d]	12.75[b]	3.82[c]	50.13[a]	1.65[c]
F3 extract	43.9[b]	5.77[c]	4.35[b]	1.24[b]	3.07[e]	15.30[c]	9.50[c]	29.19[a]	29.13[b]	2.45[b]
F4 extract	23.3[b]	14.46[b]	1.92[c]	0.64[c]	0.83[f]	35.68[b]	9.61[c]	26.03[b]	6.52[d]	4.32[a]
F4 residue	181.2[a]	4.37[d]	1.20[d]	0.25[d]	2.17[e]	82.28[a]	1.96[d]	0.57[d]	6.90[d]	0.30[d]
Two way ANOVA results P[‖]										
°Brix[#]	ns	<0.001	ns	<0.01	ns	ns	<0.001	ns	<0.01	<0.001
Fraction	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
°Brix x Fraction	ns	<0.01	<0.001	ns	<0.001	ns	ns	ns	<0.05	<0.001

[†] Monosaccharide composition expressed as molar percentage with abbreviations Man—mannose, Rha—rhamnose, GlcA—glucuronic acid, GalA—galacturonic acid, Glc—glucose, Gal—galactose, Xyl—xylose, Ara—arabinose, Fuc—fucose.
[‡] Cell wall material (CWM) extracts following sequential fractionation of whole CWM F1 to F4, and the recovered F4 CWM residue followng 4 M KOH extraction.
[‖] Significance by 2-way ANOVA: $n = 30$; ns—not significant; differences between means indicated by different letters within a column using a post-hoc Tukey's HSD test.
[#] Two ripeness stages, 23 °Brix or 25 °Brix).

of other non-polysaccharide material, particularly in F1 and F2, may have contributed by weight. The strong likelihood that lignin was co-extracted with hemicelluloses during the fractionation procedure has been mentioned previously. In our previous publications describing composition of CWMs prepared using the same buffered-phenol method, higher lignin values were determined without fractionation (Bindon et al., 2012; Bindon & Smith, 2013) than when subjected to fractionation with detergents (Bindon, Smith, & Kennedy, 2010), and also showed significant contributions of cell wall-bound proanthocyanidin, protein and inorganic ions by mass. However, in extracts obtained following sequential fractionation, no solubilised protein was detectable (data not shown) using a fluorescence-based method (EZQ protein quantitation kit, Invitrogen, Mt Waverley, VIC, Australia). The presence of proanthocyanidin in the respective fractions will be discussed in a later section.

3.2. Compositional analysis of monosaccharides in cell wall fractions

The molar proportion of monosaccharides (Tables 2 and 3) following acid-hydrolysis was used to derive information regarding the primary classes of cell wall polysaccharides within each fraction. A graphical representation of this data is shown as supporting information (Supporting information S1). The statistical analysis of the sample set showed only minor differences in monosaccharide composition between the samples from the two grape varieties and ripeness stages studied, with the greatest differences observed by fraction.

For the F1 extract, galacturonic acid was the greatest by molar proportion (43 to 45%), followed by arabinose and galactose. This is indicative that this fraction contained primarily pectic polysaccharides: homogalacturonan and potentially some

Table 3
Monosaccharide recovery and composition for cell wall fractions of Shiraz grape skins at two ripeness stages.

	Total yield	Monosaccharide composition (mole %)[†]								
	(mg/g F0)	Man	Rha	GlcA	GalA	Glc	Gal	Xyl	Ara	Fuc
23 °Brix										
F1 extract[‡]	22.9[c]	1.68[f]	3.40[c]	2.89[a]	44.65[a]	2.48[e]	17.66[a]	4.21[de]	21.11[c]	1.92[bc]
F2 extract	21.5[c]	1.10[f]	7.97[a]	3.37[a]	14.96[b]	5.83[d]	15.52[b]	5.24[d]	44.48[a]	1.53[c]
F3 extract	49.8[b]	5.64[cd]	4.09[bc]	1.17[b]	2.91[d]	14.79[c]	10.63[d]	27.09[ab]	31.08[b]	2.60[b]
F4 extract	27.0[bc]	15.70[a]	1.07[e]	0.77[bc]	0.94[e]	36.49[b]	10.40[d]	25.75[bc]	4.96[e]	4.48[a]
F4 residue	177.3[a]	4.63[de]	1.14[de]	0.24[d]	2.26[de]	81.37[a]	2.22[f]	0.53[f]	7.11[de]	0.50[d]
25 °Brix										
F1 extract	23.2[c]	1.55[f]	3.83[bc]	3.26[a]	42.73[a]	2.24[e]	18.03[a]	3.29[e]	22.45[c]	2.62[b]
F2 extract	23.2[c]	1.04[f]	7.72[a]	3.10[a]	12.57[c]	8.53[d]	14.21[c]	4.71[de]	46.45[a]	1.66[c]
F3 extract	45.8[bc]	6.00[c]	4.24[b]	1.21[b]	2.70[d]	16.73[c]	9.41[e]	27.87[a]	29.52[b]	2.31[bc]
F4 extract	25.7[bc]	13.79[b]	1.82[d]	0.49[cd]	0.38[de]	35.24[b]	10.23[de]	24.79[c]	8.28[d]	4.41[a]
F4 residue	177.9[a]	4.44[e]	1.26[de]	0.23[d]	2.21[de]	81.60[a]	2.13[f]	0.56[f]	7.24[de]	0.33[d]
Two way ANOVA results P[‖]:										
°Brix[#]	ns	<0.05	<0.05	ns	<0.01	ns	<0.001	ns	<0.05	ns
Fraction	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
°Brix × fraction	ns	<0.001	<0.05	<0.05	<0.01	<0.05	<0.001	ns	<0.05	ns

[†] Monosaccharide composition expressed as molar percentage with abbreviations Man—mannose, Rha—rhamnose, GlcA—glucuronic acid, GalA—galacturonic acid, Glc—glucose, Gal—galactose, Xyl—xylose, Ara—arabinose, Fuc—fucose;
[‡] Cell wall material (CWM) extracts following sequential fractionation of whole CWM F1 to F4, and the recovered F4 CWM residue followng 4 M KOH extraction;
[‖] Significance by 2-way ANOVA: $n = 30$; ns—not significant; differences between means indicated by different letters within a column using a post-hoc Tukey's HSD test.
[#] Two ripeness stages, 23 °Brix or 25 °Brix).

rhamnogalacturonan I. It also potentially contained a significant contribution of arabinogalactan Type I and arabinan. The identification of the principal arabinogalactan is based on previous studies of the varieties under investigation, which have used linkage analysis to show that Type I arabinogalactan is more abundant than Type II, even at late-stage ripening (Bindon et al., 2012; Vicens et al., 2009). A minor contribution of xylose (±4%) may indicate a trace of xyloglucan.

For the F2 extract, arabinose formed the greatest component by molar percentage, indicating that the bulk of polysaccharide removed was likely to be arabinan. A significant quantity of galactose, may indicate the presence of further arabinogalactan Type I, although less abundant than in the F1 samples. The increased ratio of galacturonic acid to rhamnose, glucuronic acid and fucose in F2 relative to F1 potentially shows that the proportion of rhamnogalacturonan to homogalacturonan was increased in the pectic component of the polysaccharide.

Further drops in galacturonic acid by molar proportion were found with the sequential fractionation steps, indicating that the bulk of pectic polysaccharides were liberated in F1 and F2. The F3 extract had proportionally higher xylose and arabinose than the other sugars, and these were equimolar, which may point to a high contribution of arabinoxylan (glucurono(arabino)xylan). The presence of higher proportions of glucose and mannose by comparison with F1 and F2, while maintaining a relatively high proportion of galactose indicates potential contributions of arabinogalactan Type I, arabinan, heteromannan and xyloglucan to the polysaccharide matrix. By comparison, glucose and xylose formed the bulk of the F4 extract, indicating the presence of xyloglucan, which is expected based on work done in grape mesocarp (Nunan et al., 1998) and grapevine leaves (Moore et al., 2014). However, the presence of a significant quantity of mannose by comparison with the previous fractions shows that the highest proportion of heteromannan was in this extract. From other studies, CDTA extracts followed by Na_2CO_3 extraction was found to sequentially remove heteromannans from Muscat Gordo Blanco grape mesocarp walls (Nunan et al., 1998). In the current study on grape skin cell walls, the bulk of heteromannan appeared to be liberated only at higher concentrations of KOH, indicating a stronger association with the cell wall.

Based on published research (Nunan et al., 1998) it would be expected that the residue following fractionation with 4 M KOH is primarily cellulose. Hydrolysis of the F4 residue in harsh acid yielded >80% of glucose by molar proportion, with minor contributions of mannose and arabinose. We suggest that this is strong evidence that the polysaccharide composition of residues following sequential fractionation was chiefly cellulose, with residual bound heteromannan and arabinan.

3.3. Concentration and composition of acetone-extractable and cell-wall bound skin proanthocyanidin

The cell wall fractions were further analysed for proanthocyanidin concentration and composition using methods developed for the direct analysis of fibres. Since KOH was selected as the fractionation solvent, the potential oxidation of cell wall bound proanthocyanidin was an important consideration in applying these methods (Cilliers & Singleton, 1990). As outlined earlier in this report, it was found that significant reduction in the conversion of proanthocyanidin to anthocyanidin in the butanol–HCl assay could be expected after exposure to KOH. Based on work with a purified skin tannin standard, the predicted proanthocyanidin concentration after KOH treatment using the modified butanol–HCl assay would be in the order of 40% of a control sample. As such, the data generated using this method was assumed to indicate the presence of residual proanthocyanidin in the KOH-extracted fractions, but is not quantitative.

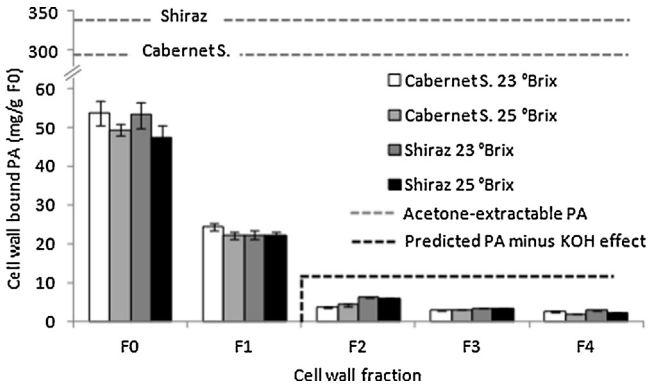

Fig. 3. Analysis of cell wall fractions from two grape varieties and ripeness stages for bound proanthocyanidin using a modified HCl–butanol assay where data are given as mean and standard error of three replicates. The point of oxidation by KOH is indicated, thereafter a response using the assay indicates the presence of proanthocyanidin but does not give quantitative information.

The acetone-extractable proanthocyanidin yield was high by comparison with other seasons for the same vineyard (Bindon et al., 2012; Bindon & Kennedy, 2011) and on a skin cell wall dry weight basis was ± 290 mg/g for Cabernet Sauvignon, and ±320 mg/g for Shiraz (Fig. 3). Only minor differences between grape samples were found in terms of ripeness grade. It was found that the cell wall-bound proanthocyanidin concentration did not change significantly during the buffered phenol procedure (data not shown). Therefore, cell-wall bound proanthocyanidin data was expressed per unit of purified cell wall (F0) (Fig. 3). The proportion of cell wall-bound proanthocyanidin was 13% to 16% of total grape skin proanthocyanidin (acetone-extractable + cell wall-bound). Following extraction in CDTA, a significant (54% to 58%) drop in cell wall-bound proanthocyanidin was observed (Fig. 3). After extraction with KOH, a further significant decrease in cell wall-bound proanthocyanidin occurred, however we expected that the KOH extraction step would have reduced colour development using the HCl–butanol assay. Nevertheless, the results indicate that there was a residual fraction of proanthocyanidin associated with hemicelluloses and cellulose cell wall residues, which has not yet been shown for grape skin cell wall material.

To observe changes in proanthocyanidin composition following extraction with CDTA, phloroglucinolysis was performed on cell wall residues of F0 and F1, and compared with the acetone-extractable proanthocyanidin component (Table 4). Due to either to KOH exposure, or low residual proanthocyanidin, no signal was obtained using phloroglucinolysis in F2 to F4. The acetone-extractable Cabernet Sauvignon grape skin proanthocyanidin was found to have a high molecular mass, exceeding an mDP of 40 units. This, together with the proanthocyanidin concentration was far higher than observed in previous seasons for samples taken from the same grapevines (Bindon & Kennedy, 2011). This indicates that seasonal variability may strongly drive differences in both proanthocyanidin concentration and composition, and the factors which may drive this are poorly understood at present. The cell wall-bound proanthocyanidin in F0 and F1 Cabernet Sauvignon was similar to the acetone-extractable fraction in terms of mDP, but differences were found in composition, having proportionally lower epigallocatechin, and proportionally higher catechin, epicatechin and epicatechin-gallate as extension units. Epicatechin as a terminal unit was not detected in F0 and F1, but we note that this may be due to its low relative proportion, being simply below the detection limit of the method.

The mDP of the acetone-extractable Shiraz proanthocyanidin was below that of Cabernet Sauvignon, and was higher in the F1 fraction of the 23 °Brix sample, although this was not statistically

Table 4
Proanthocyanidin subunit composition for determined by direct phloroglucinolysis on a purified acetone-extractable isolate, total cell wall material (F0), and CDTA-insoluble residues (F1) prepared from Cabernet Sauvignon and Shiraz grape skins at two ripeness stages.

	Cabernet Sauvignon								Shiraz							
	mDP[†]	MM[‡]	Extension (%)[‖]				Terminal (%)[‖]		mDP[†]	MM[‡]	Extension (%)[‖]				Terminal (%)[‖]	
		Subunit	EGC	C	E	ECG	C	E		Subunit	EGC	C	E	ECG	C	E
Acetone extraction (ACE)																
23 °Brix	42.59	12,846	54.06^{a}	1.42^{b}	41.49^{b}	3.02^{b}	83.4^{b}	16.60	30.12^{b}	9114^{b}	42.07^{a}	1.49^{c}	50.61^{c}	5.82^{c}	84.8^{b}	15.2^{d}
25 °Brix	48.17	14,533	53.68^{a}	1.31^{b}	41.93^{ab}	3.07^{b}	82.8^{b}	17.20	29.98^{b}	9082^{b}	42.41^{a}	1.39^{c}	50.11^{c}	6.07^{c}	80.6^{c}	19.3^{c}
Purified CWM (F0)[#]																
23 °Brix	52.25	15,724	45.84^{ab}	2.26^{ab}	48.36^{ab}	3.54^{a}	100.0^{a}	nd	33.33^{b}	10,062^{b}	28.30^{b}	2.83^{ab}	61.67^{b}	7.18^{b}	75.5^{d}	24.5^{b}
25 °Brix	44.60	13,463	48.69^{b}	3.29^{a}	43.85^{ab}	4.16^{a}	100.0^{a}	nd	28.42^{b}	8582^{b}	28.09^{b}	3.02^{a}	61.59^{b}	7.29^{b}	66.9^{e}	33.1^{a}
CDTA-insoluble CWM (F1)[#]																
23 °Brix	40.93	12,330	43.96^{b}	2.91^{a}	49.00^{ab}	4.12^{a}	100.0^{a}	nd	47.58^{a}	14,387^{a}	28.36^{b}	2.41^{b}	61.69^{b}	7.52^{ab}	100.0^{a}	nd
25 °Brix	44.85	13,498	43.21^{b}	2.47^{ab}	50.31^{a}	4.00^{a}	100.0^{a}	nd	39.73^{ab}	12,019^{ab}	24.15^{c}	2.88^{ab}	64.65^{a}	8.31^{a}	100.0^{a}	nd
Two way ANOVA results P[§]																
°Brix[♦]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	<0.01	ns	<0.05	<0.05	<0.001	<0.001
Fraction (ACE, F0, F1)	ns	ns	<0.001	<0.001	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
°Brix × fraction	ns	ns	ns	<0.05	ns	ns	ns	ns	ns	ns	<0.001	ns	<0.001	ns	<0.001	<0.001

[†] Mean degree of polymerisation in epicatechin units.

[‡] Molecular mass as determined by phloroglucinolysis.

[‖] Percent composition of proanthocyanidin subunits (in mg) of extension or terminal subunits with the following subunit abbreviations: EGC, (−)-epigallocatechin; C, (+)-catechin; EC, (−)-epicatechin; ECG, (−)-epicatechin-3-*O*-gallate. Terminal (−)-epicatechin-3-*O*-gallate was not detected in samples.

[#] CWM—cell wall material.

[§] Significance by 2-way ANOVA: $n = 18$; ns—not significant; differences between means indicated by different letters within a column using a post-hoc Tukey's HSD test.

[♦] Two ripeness stages, 23 °Brix or 25 °Brix); nd—not detected.

Table 5
Adsorption of proanthocyanidin by whole cell wall material (F0), and cell wall residues following fractionation (F1 to F4) from Cabernet Sauvignon and Shiraz grape skins at two ripeness stages.

	Proanthocyanidin adsorbed per fraction					
	Cabernet Sauvignon			Shiraz		
	(mg/g F0)[†]	(mg/g F_n)[‡]	(mg/g poly)[‖]	(mg/g F0)[†]	(mg/g F_n)[‡]	(mg/g poly)[‖]
23 °Brix						
F0[#]	153^{a}	153^{bcd}	520^{a}	168^{a}	168^{c}	563^{a}
F1	112^{b}	148^{cd}	384^{b}	105^{bc}	136de	383^{b}
F2	102^{bc}	198^{a}	353^{b}	113^{b}	227^{a}	446^{b}
F3	66^{d}	169^{abc}	224^{c}	73^{d}	179^{bc}	362^{b}
F4	35^{e}	112^{e}	120^{d}	47^{e}	136de	264^{c}
25 °Brix						
F0	152^{a}	152^{bcd}	505^{a}	166^{a}	166^{cd}	564^{a}
F1	112^{b}	149^{cd}	378^{b}	103^{c}	136^{e}	382^{b}
F2	95^{c}	184^{ab}	323^{b}	102^{c}	202^{ab}	412^{b}
F3	71^{d}	178^{abc}	238^{c}	74^{d}	186^{bc}	365^{b}
F4	38^{e}	123de	128^{d}	38^{f}	134^{e}	214^{c}
Two way ANOVA results P[§]						
°Brix[♦]	ns	ns	ns	<0.001	ns	ns
Fraction	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
°Brix × fraction	ns	ns	ns	<0.01	ns	ns

[†] Proanthocyanidin (PA) adsorbed from a 2 mg/mL control solution by whole CWM F0 and by the CWM residues F1 to F4 following fractionation, expressed as mg PA per 10 mg of F0 pre-fractionation.
[‡] PA adsorption expressed per mg gravimetric recovery of each fraction (F_n).
[‖] PA adsorbed per g of monosaccharide expected within the residue based on the sum quantified from extracts and F4 residue.
[#] F0—whole unfractionated CWM and CWM residues collected following sequential fractionation F1 to F4 (residues).
[§] Significance by 2-way ANOVA: $n = 30$; ns—not significant; differences between means indicated by different letters within a column using a post-hoc Tukey's HSD test.
[♦] Two ripeness stages, 23 °Brix or 25 °Brix).

significant for the riper, 25 °Brix sample. From the statistical analysis, the effect of fractionation on proanthocyanidin composition was greater than grape ripeness. Thus, for most compositional parameters measured only minor changes were found by ripeness grade. Like the Cabernet Sauvignon samples, the proanthocyanidin bound to F0 and F1 Shiraz fractions showed larger changes in subunit composition than mDP. The Shiraz proanthocyanidins had lower extension epigallocatechin than the Cabernet Sauvignon samples, and higher epicatechin and epicatechin gallate as extension units. In a like manner to the Cabernet Sauvignon cell wall fractionation experiment, extension epigallocatechin was proportionally lower in F0 and F1 residues than in the acetone-extractable fraction, and the remaining extension subunits proportionally higher.

Our previous reports found greater differences in proanthocyanidin mDP between the acetone-extractable fraction and that bound to cell walls, being higher in the bound fraction (Bindon et al., 2012; Bindon, Smith, & Kennedy, 2010). As mentioned previously, this highlights that a large variability exists. The current data suggest that there was a greater selectivity for proanthocyanidin enriched in epicatechin, catechin and epicatechin-gallate as extension units in the cell wall-bound fraction, when changes in proanthocyanidin molecular mass are not considered. By comparison, proanthocyanidin containing a higher proportion of epigallocatechin (as extension units) would appear to be more extractable in acetone.

3.4. Binding properties of cell wall fractions for proanthocyanidin

Given that a significant loss of proanthocyanidin was found following CDTA treatment, concomitant with the removal of galacturonan-rich pectic polysaccharides (Tables 2 and 3, Fig. 3) it appears that the bulk of cell wall-bound proanthocyanidin was associated with a relatively minor component of the cell wall. It was of interest to determine whether a similar effect could be seen in the binding capacity of grape cell wall residues for proanthocyanidin following fractionation.

It was necessary to consider the binding capacity of the fractions both as a proportion of total cell wall material (F0) and in terms of the quantity of material removed after each extraction (F_n). Since different quantities were removed for each fraction (as monosaccharide units), the binding capacity per fraction could also be expressed as units bound per unit of residual polysaccharide (Table 5). Statistical analysis indicated that the greatest effect on binding capacity was due to the fractionation, rather than grape ripeness grade. This would be expected based on the observations regarding proanthocyanidin (residues) and monosaccharide (extracts and residues) composition which were discussed previously.

The proanthocyanidin binding capacity of F0 cell wall material was high by comparison with previous studies on the skin cell wall and tannin fractions prepared from the same vineyard, and assayed under the same conditions (Bindon et al., 2012). This highlights once again that great seasonal variability in sample composition exists which could impact upon processing outcomes.

When proanthocyanidin binding capacity per fraction was expressed as a proportion of cell wall starting material (10 mg F0), it was found that the greatest reduction in binding capacity occurred following the removal of galacturonan-rich pectic polysaccharides, independently of grape variety or ripeness stage. However, expressing proanthocyanidin binding capacity in terms of gravimetric recovery of the residue (F_n) or as mg polysaccharide remaining in the residue showed only a minor change from F0 to F1, which was significant for Shiraz but not Cabernet Sauvignon grapes. The varietal difference could not be explained by differences in polysaccharide composition, or the quantity of cell wall material removed by CDTA, as these were similar between samples. The conversion to polysaccharide by mass within the CDTA-soluble fraction was low (Tables 2 and 3), which meant that there was a significant contribution of non-polysaccharide material to the F1 extract. Expressing the binding capacity of the cell wall for proanthocyanidin as polysaccharide units gave a very high value for F0, indicating that for only a minor loss of pectin, there was a significant drop in binding capacity by the polysaccharides in the F1 residue. The main conclusion drawn from this observation is that while a

significant loss of proanthocyanidin binding capacity occurred after CDTA extraction, this fraction was a significant component of the cell wall by weight (Table 1) but minor in terms of overall polysaccharide. This shows the high propensity for proanthocyanidin to associate with the galacturonan-rich fraction of the cell walls, and lends further support to the data presented in Fig. 3, which shows a significant drop in cell-wall bound proanthocyanidin with removal of F1.

Various studies have shown that a stronger adsorption of proanthocyanidin might be expected within the pectin-rich cell wall fraction, in particular more highly methylated galacturonans (Le Bourvellec et al., 2005, 2009, 2012; Watrelot et al., 2013; Watrelot, Le Bourvellec, Imberty, & Renard, 2014). Specifically comparing the role of neutral pectic side chains ('hairy' regions), these appear to reduce proanthocyanidin–polysaccharide interactions, independent of galacturonic acid content (Watrelot et al., 2014). In particular, low affinities have been observed for arabinogalactans (Watrelot et al., 2014). In the current study, the CDTA-extractable component was enriched in galacturonan relative to arabinogalactan, but overall the galacturonan of the sample would be expected to have a low degree of methylation (Fig. 2B) by comparison with that reported in literature for the same grape varieties (Ortega-Regules, Ros-García, Bautista-Ortín, López-Roca, & Gómez-Plaza, 2008; Vicens et al., 2009). It is possible that a low degree of methylation could have limited the interaction of proanthocyanidin with this fraction of the cell wall (Watrelot et al., 2013). Considering that there was also a significant loss of proanthocyanidin associated with this fraction, it cannot be ruled out that potential binding sites for proanthocyanidin were already saturated.

In summary, the data show that the CDTA-extractable component represents a significant fraction of the cell wall in terms of its overall binding capacity for proanthocyanidin. However, the results also indicate that the bulk of the cell wall retains a relatively high proanthocyanidin binding capacity, independent of the contribution of the pectic fraction. This is in strong agreement with work done on insoluble apple cell walls, where the modification of cell wall composition by the selective removal of a pectic-rich polysaccharide fraction (by boiling) reduced the affinity of apple cell walls for proanthocyanidin (Le Bourvellec et al., 2012) but it was found that the residue nonetheless retained a high binding capacity for proanthocyanidin.

The binding capacity of the F1 residue was further characterised following sequential fractionation in KOH. After extraction in 50 mM KOH, the change in proanthocyanidin binding capacity from F1 to F2 expressed either as a proportion of F0, or in polysaccharide units was negligible. On a gravimetric basis (F2), however, the binding capacity appeared to increase from F1 to F2. This was due to a 24% to 28% loss of cell wall material with the F2 extraction by weight, which was low in polysaccharide. As for the F1 extraction, this may indicate that there was a significant contribution of non-polysaccharide material in the F2 extract which did not bind to, or was saturated with bound proanthocyanidin. The decrease in 550 nm absorbance in the butanol–HCl assay of the F2 residues (Fig. 3) may indicate that a loss of proanthocyanidin occurred, but this remains speculative due to potential oxidation events in the presence of KOH (Cilliers & Singleton, 1990). A further possibility is that the non-polysaccharide material removed was enriched with lignin, as discussed previously. The 50 mM KOH extraction may therefore have made more binding sites available to proanthocyanidin within the residue, consisting primarily of arabinoxylan, xyloglucan and cellulose. This may have increased the binding capacity when expressed per unit of cell wall residue (F_n).

Further significant losses in proanthocyanidin binding capacity were observed with sequential extraction in 1 M and 4 M KOH. As such, proanthocyanidin binding capacity expressed in terms of mg F0 was lowest in F3, followed by F4. However, considering the low amount of cell wall material (cellulose and lignin) remaining after these extractions, the binding capacity of both F3 and F4 expressed as F_n or as polysaccharide units was higher than expected. The binding capacity of F3 was higher than F4, independently of the units of expression, which may indicate that the combination of xyloglucan and heteromannan enhanced proanthocyanidin adsorption within the cellulose matrix. This result is not unexpected based on published work using model polysaccharides, which has shown that cross-linked xyloglucan has a higher affinity for proanthocyanidin than cellulose (Le Bourvellec et al., 2005).

For the current dataset, the selectivity of proanthocyanidin binding was compared following reaction of each fraction with the control by looking at changes in the molecular mass distribution by gel permeation chromatography. The measurement of proanthocyanidin mDP provides limited information on the selectivity of binding, and the result is highly dependent upon the concentration of proanthocyanidin remaining in solution. Analysis of changes across the entire molecular mass distribution affords a better sensitivity, and less error in interpretation of the results. Fig. 4 shows the quantum of change in proanthocyanidin molecular mass within different molecular mass categories where 10% elution represents the lowest, and 90% elution the highest. Due to similarity between the samples tested, the specific sample information within each cell wall fraction was not presented. For all cell wall fractions tested, there was an overall decrease in proanthocyanidin molecular mass as evidenced by their position on the *x* axis. With the exception of F4, all fractions showed stronger decreases in the high molecular mass range, indicating a selectivity in proanthocyandin binding. Due to the fact that the concentration decrease in supernatants after being reacted with the respective fractions was different, this was included to further qualify the data presented (*y*-axis). It is evident that with a smaller change in proanthocyanidin concentration after adsorption by a given cell wall fraction, the quantum of change in molecular mass was smaller. This is most likely to be a concentration-dependent effect, and limits further interpretation of the data. Therefore, except in the case of F4 where more scatter was observed within the data, the results suggest that all cell wall fractions displayed a preference for the highest molecular mass proanthocyanidins. This confirms the findings of Le Bourvellec et al. (2005) who in a previous study suggested that there is little difference in the selectivity of hemicelluloses for proanthocyanidin type as a function of their composition. For cellulose, it was proposed that a low affinity for proanthocyanidin may be due to a low porosity, restricting the point of interaction to a surface phenomenon. It would appear that this confers a poor selectivity of binding in terms of proanthocyanidin molecular mass (Le Bourvellec et al., 2005).

3.5. The potential effect of the drying method on cell wall three-dimensional structure and proanthocyanidin binding properties

The three-dimensional structure of cell wall confers their relative porosity, which is in turn correlated with surface area available for binding events to occur. The approach used to dry cell wall preparations can impact upon the porosity of the cell wall (Guillon, Auffret, Robertson, Thibault, & Barry, 1998; Le Bourvellec & Renard, 2005; Le Bourvellec et al., 2012; Karathanos, Anglea, & Karel, 1996; Krokida, Karathanos, & Maroulis, 1998). An observation by Guillon et al. (1998) was that harsh drying (100 °C) of sugar beet fibres did not greatly impact upon porosity. However, following the extraction of pectic polysaccharides, harsh drying significantly reduces fibre porosity, allowing the suggestion that the presence of pectin within the cell wall structure protects it from collapse during drying. Furthermore, Guillon et al. (1998) reported that lyophilisation of sugar beet fibres after removal of pectins resulted in no change in cell wall porosity in comparison with native fibres.

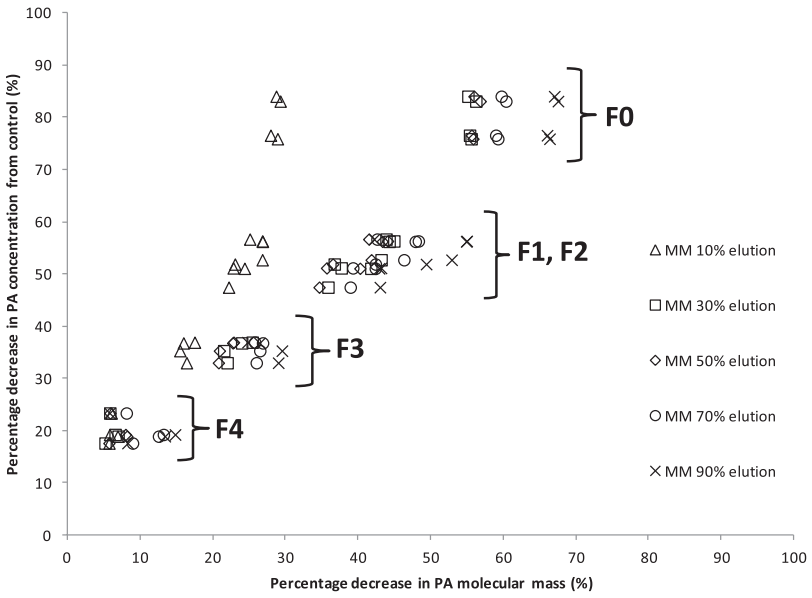

Fig. 4. Change in proanthocyanidin molecular mass as a function of proanthocyanidin concentration decrease after reaction of a 2 g/L skin proanthocyanidin solution with whole cell wall material (F0) and cell wall fractions (F1 to F4). Molecular mass differences were determined by gel permeation chromatography where elution cut-off points represent the lowest (10% elution) to highest (90% elution) quantified molecular mass.

Various studies have shown that maintaining the temperature during lyophilisation below the glass transition point (−45 °C) is important in preventing cell wall collapse and shrinkage during drying, and maintaining porosity (Karathanos et al., 1996; Krokida et al., 1998). However, in comparative studies of various tissues, apple tissue was noted be prone to cell wall collapse in a temperature-dependent manner as lyophilisation temperatures increased above glass transition point, resulting in decreased porosity and bulk density. In line with this observation, apple cell wall preparations from which pectic polysaccharides were removed by boiling had higher porosity when dried by solvent exchange (3.16 m^2/g) in comparison with lyophilisation (1.26 m^2/g) (Le Bourvellec et al., 2012).

In light of these observations, a critique of the current study is that the samples were prepared *via* lyophilisation instead of solvent exchange, could have resulted in structural collapse of the cell wall. This is important, since the reduction of apple cell wall porosity by harsh drying (100 °C) significantly decreases cell wall affinity for proanthocyanidin, in particular proanthocyanidin of higher molecular mass (Le Bourvellec & Renard, 2005). Therefore, in interpreting the results of the current study, it cannot be excluded that drops in proanthocyanidin binding capacity following sequential removal of polysaccharide classes may have been due to the combined effect of polysaccharide removal and a possible reduction in porosity as a result of the lyophilisation steps.

Currently, the effect of drying method on grape skin cell wall porosity is unknown, and it is also unclear whether grape tissue is prone to cell wall collapse during lyophilisation in the same way as apple tissue. However, given that our cell wall samples were pretreated in 70% acetone (i.e. low water content) and lyophilised at low temperature (≤−50 °C), it is possible that changes in porosity were minimal, or genuinely induced by the removal of polysaccharide components. In our previous work on commercially-ripe grape cell walls prepared using the same lyophilisation approach as in the current study, surface area (porosity) determined using nitrogen adsorption ranged from 2.42 to 2.89 m^2/g (Bindon et al., 2014). These values are similar to the surface area determined for apple cell wall preparations following solvent exchange, of 2.15 to 2.64 m^2/g (Le Bourvellec et al., 2012). Small sample sizes prevented porosity measurements in cell walls and sub-fractions the current study.

Irrespective of the porosity of the cell wall starting material, a key objective of our study was to determine the effect of polysaccharide extraction on cell wall binding properties for proanthocyanidin. It is noteworthy that in the study by Le Bourvellec et al. (2012), despite a reduced porosity in boiled apple cell wall material which had been lyophilised, cell wall affinity for proanthocyanidin on a gravimetric basis was unchanged in comparison with the same material of a higher porosity, which had undergone solvent-exchange drying. In fact, the cell walls dried by solvent-exchange had increased porosity relative to the original preparation due to the removal of pectins. This is different from what would be expected based on the earlier studies by this group in which harsher drying, and lower porosity in the cell wall preparation reduced its affinity for proanthocyanidin (Le Bourvellec & Renard, 2005). This result indicated a strong relationship between pectic polysaccharide composition and content in conferring cell wall affinity for proanthocyanidin.

Therefore, it would appear that while cell wall porosity may be important in defining the proanthocyanidin-binding response under certain conditions (Le Bourvellec & Renard, 2005; Bindon et al., 2014), the compositional characteristics which confer proanthocyanidin binding sites within cell walls is of equal significance, in particular with regard to the effect of processing on polysaccharide distribution in the cell wall (Le Bourvellec et al., 2012). For the current study, the removal of the CDTA-soluble fraction of the cell wall was found to most significantly reduce proanthocyanidin binding by cell walls, which is in strong agreement with the results on apple (Le Bourvellec et al., 2012).

3.6. General conclusions and commercial implications of the results

The current data support the idea that the pectic fraction represents a significant component of the cell wall in terms of binding capacity for proanthocyanidin. It was noteworthy, however, that a high residual binding capacity was found for hemicellulose. Cellulose (and residual lignin), which represents approximately 30% of the cell wall by weight had a low adsorption capacity for proanthocyanidin. The results have important commercial implications, since it has been demonstrated that the interaction between cell

walls and proanthocyanidin is a key limiting factor in proanthocyanidin solubility, both for grape (Bindon, Smith, & Kennedy, 2010) and other commercial crops (Grabber et al., 2013; Le Bourvellec et al., 2007; Perez-Jimenez & Torres, 2011). This defines the point at which the cell wall structure may be manipulated to significantly alter the extraction of proanthocyanidins associated with cell walls, and potentially limit their re-adsorption following solubilisation. This might be strategically applied in the case of commercial operations which require the transfer of proanthocyanidin into solution, as in the case of wine or cider production.

Published work has shown that while a significant amount of grape skin proanthocyanin is bound to cell walls, and non-extractable even with a harsh solvent (70% v/v acetone), under the conditions of fermentation only a fraction of the total skin tannin is extracted into wine (Bindon, Smith, & Kennedy, 2010). For the current dataset, the model binding experiments are therefore of more relevance to vinification conditions than are the data for non-extractable (cell wall-bound) proanthocyanidin. This is because the cell walls remain saturated with proanthocyanidin during the extraction phase of vinification. From these experiments, it is evident that techniques which manipulate (or degrade) the pectic (CDTA-extractable) or hemicellulosic fractions are likely to render proanthocyanidin more extractable during fermentation.

A possibility to induce breakdown of cell wall structure is the application of enzymes, and these are widely available for commercial use. Indeed, the application of enzymes with polygalacturonase and beta-glucanase (Ducasse et al., 2010) activity can increase the extraction of proanthocyanidin to wine, while at the same time enriching the polysaccharide profile with rhamnogalacturonan and reducing the contribution of arabinogalactans. A number of points should be considered with the applications that might inhibit their effectiveness, and which require further research. Access of enzymes to cell wall residues for cleavage by enzyme activity may be restricted by the presence of proanthocyanidin, either directly bound or indirectly associated as multilayers (Le Bourvellec et al., 2009). The presence of soluble polysaccharide has also been observed to desorb bound proanthocyanidin from cell walls (Renard et al., 2001) and enzyme application may in fact reverse this beneficial effect by breaking down solubilised polysaccharides.

A further technique which can enhance both proanthocyanidin and polysaccharide extraction (grape-derived arabinogalactans and rhamnogalacturonan) is flash treatment (Doco et al., 2007; Morel-Salmi et al., 2006). However, this is effective only when flash-treated musts are fermented with skin contact. This indicates that the extraction of both proanthocyanin and polysaccharide are via diffusion from the grape skins, and this has been confirmed through studies on red wine polysaccharide evolution during fermentation (Guadalupe & Ayestaran, 2007).

The choice of yeast for fermentation can also alter the extraction of proanthocyanidin (Holt et al., 2013) but interestingly, it has been found that yeast strains with specific polygalacturonase activity did not significantly affect wine proanthocyanidin concentration (Eschstruth & Divol, 2011). A greater understanding of vinification techniques, promising new technologies such as pulsed electric field (Puertolas, Saldana, Condon, Alvarez, & Raso, 2009; Cholet et al., 2014) and microwave-assisted extraction is required, as these may hold promise as tools to optimise proanthocyanidin and polysaccharide extraction into wine through targeted breakdown of the skin cell wall structure.

While improving proanthocyanidin extraction may be desirable in many circumstances, it is well known that at high wine proanthocyanidin concentrations, clarity problems or excessive astringency can occur, introducing the requirement for fining (Bindon & Smith, 2013). While this has traditionally been done using proteinaceous fining agents, recent concerns regarding the use of animal-based products in wine, which are possibly allergenic in nature, has highlighted the need for alternative products to be developed (Bindon & Smith, 2013). We have recently identified the application of insoluble fibres as a promising new fining agent, as it selectively removes wine proanthocyanidin in a dose-specific manner (Bindon & Smith, 2013). The results of the current study highlight that the identification and application of pectin-rich cell wall sources may be a means to improve the functionality of insoluble fibres as alternative fining agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.07.024.

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