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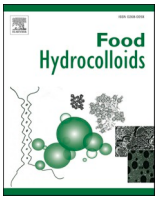

Pectolytic enzyme treatment partially degrades an arabinogalactan protein–rhamnogalacturonan I–xyloglucan co-polymer in red wine as characterised using epitope mass profiling

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ABSTRACT

Polysaccharides are important metabolites in red wine but are challenging to separate from yeast-derived mannoproteins, and costly to identify using sialylation and gas chromatography. Affinity and gel permeation chromatography was combined with immunodetection of various specific plant cell wall polysaccharide epitopes to understand this complex mixture of extracted cell wall polymers. A survey of Cabernet Sauvignon wines produced with and without pectolytic enzymes suggested that enzyme-treated wines have fewer polysaccharides in the 85–105 kDa and 1050–6000 kDa ranges. When yeast-derived mannoproteins are excluded (15.3% of total), pectolytic enzyme treatment was shown to alter the molecular weight wine polysaccharide composition between the 100–1000 kDa ranges. Furthermore, ELISA data suggested that many of the soluble polysaccharides in the 300–1000 kDa range are an arabinogalactan protein–rhamnogalacturonan I–xyloglucan co-polymer. This is the first report to use ELISA to identify changes in specific polysaccharide classes during enzyme preparation used in wine maceration.

1. Introduction

Polysaccharides in red wine are a complex mixture of compounds derived from grape cell walls and release by yeast walls during alcoholic fermentation and during ageing on the lees by cell autolysis; this mixture is determined by grape varietal, vineyard conditions, winemaking practices, and ageing. The major components of red wine include water, ethanol, acids, polyphenols, sugars and polysaccharides, and trace minerals. Polysaccharides in wine systems have been reported to affect several mouth-feel properties, including lowering astringency perception, increasing fullness and mellowness, increasing perceived viscosity, and reducing perceived 'hotness', a sense of alcohol-induced harshness in the mouth (Chong, Cleary, Dokoozlian, Ford, & Fincher, 2019; Gawel, Smith, & Waters, 2016; Soares, Mateus, & de Freitas, 2012; Vidal et al., 2004). In addition, polysaccharides have also been shown to impact a range of wine properties such as preventing protein disordering, forming colloids with polyphenols, inhibiting crystallisation, complexing with lead and interacting with aroma compounds amongst other properties (see Jones-Moore, Jelley, Marangon, & Fedrizzi, 2022). Reducing hotness and astringency and increasing mellowness are often goals of specific winemaking practices. Understanding the contribution of polysaccharides allows for more control of sensory attributes in winemaking.

Polysaccharides are polymers of monosaccharide subunits. In plants, the nine most common monosaccharides are arabinose, rhamnose, fucose, mannose, glucose, galactose, xylose, glucuronic acid, and galacturonic acid (Gao, Fangel, Willats, Vivier, & Moore, 2016; Jones-Moore et al., 2022). Polysaccharides play a structural role in the plant cell wall, and when juice is extracted from fruit, the cellular disruption causes soluble polysaccharides to dissolve into the liquid mass. In wine, the majority of polysaccharides and all simple sugars come from the crushing and release of endogenous pectinolytic enzymes of grapes during maceration and fermentation; however, yeast produce mannoproteins (MP) that are held in situ in their cell walls until lysis, after which they form a significant part of the total polysaccharide mixture (Gao et al., 2016; Vidal, Doco, Moutounet, & Pellerin, 2000). Grape

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polysaccharides include arabinogalactan proteins (AGPs), arabinans, arabinogalactans, rhamnogalacturonan type I (RG-I) and rhamnogalacturonan type II (RG-II), which are extracted by endogenous or exogenous enzymes from the pectic structures of the grape cell wall during maceration and fermentation (Vidal, Williams, Doco, Moutounet, & Pellerin, 2003). Previous research has shown that the polysaccharides in wine are composed of 37–50% AGPs, 30–45% MPs, 15–19% RG-II, and 4% RG-I (Ayestarán, Guadalupe, & León, 2004; Martinez-Lapuente et al., 2016; Vidal et al., 2003).

Enzyme maceration with exogenous pectic enzymes has been shown to change the overall quantity and composition of polysaccharides extracted into wine during fermentation. Ayesterán and co-workers (Ayestarán et al., 2004) found that enzyme use increased the concentration of all plant cell wall-derived polysaccharides. Conversely, Doco, Williams, and Cheynier (2007) and Marie-Agnès Ducasse et al. (2010) found that enzyme usage reduced arabinogalactans, AGPs, arabinans and increased RG-II. Using a glycan microarray approach coupled with monosaccharide analysis, Kuhlman, Hansen, Jørgensen, du Toit, and Moore (2022) observed a reduction in AGs, AGPs, RG-I, homogalacturonans, and xyloglucans when enzymes were utilized; however, the total amount of polysaccharide present was unaffected as measured by monosaccharide analysis.

Polysaccharides in wine systems can be measured using a few methods, but generally, it involves a precipitation step, a size separation step, and an identification step (Vidal et al., 2003). Precipitation usually is by acidic alcohol dehydration, dialysis, or ultrafiltration (Vidal et al., 2003). Size-exclusion chromatography separates the polydisperse mixture into fractions by size which can then be analysed further to determine specific polysaccharide type (Ducasse et al., 2010). Coupled with a refractive index detector (RID), a direct quantification of polysaccharide concentration can be made (Guadalupe, Martínez-Pinilla, Garrido, Carrillo, & Ayestarán, 2012). Gas chromatography coupled with flame-ionisation or mass-spectrometry is commonly used for monosaccharide analysis after acid hydrolysis and sialylation (Guadalupe, Ayestarán, Williams, & Doco, 2015). This method allows for high sensitivity towards monosaccharides. Still, it only provides an estimation of the specific polysaccharide epitopes from the ratio of monosaccharides. To further understand the structure of polysaccharides, the glycosyl-linkage composition must be determined, and even this provides only the linkage position on the sugar, not the entire structure (Pettolino, Walsh, Fincher, & Bacic, 2012).

In this work, we utilise a novel identification method for native-wine polysaccharide identification, including a size-exclusion separation coupled with an enzyme-linked immunosorbent assay method that uses monoclonal antibodies to identify well-defined plant polysaccharide epitopes (Pattathil et al., 2010; Sathitnaitham, Suttangkakul, Wonnapinij, McQueen-Mason, & Vuttipongchaikij, 2021Sathitnaitham, Suttangkakul, Wonnapinij, McQueen-Mason, & Vuttipongchaikij, 2021). Wine polysaccharides have been size-separated by other researchers and then classified using monosaccharide analysis, relying on broadly defined ratios of monosaccharides. However, polymers with heterogeneous compositions are not well defined by ratios and can only be identified using further linkage analysis, a costly and time-intensive process. This novel immunosorbent method allows rapid identification of specific classes of polysaccharides although at present limited to the 100–1000 kDa and greater range. This ELISA epitope mass profiling method has been used here for the first time to analyse both the whole wine polysaccharide extracts and those produced after pectolytic maceration enzyme treatment. These are then size fractionated at greater than 100 kDa and probed for specific plant cell wall epitopes present in a range of polysaccharides and glycoproteins. This is the first time the authors are aware that this approach has been used to characterise polysaccharides in red wine.

2. Material and methods

2.1. Reagents and equipment

Ultra-purified water refers to distilled water filtered through a Millipore Milli-Q filtration system (Millipore Merck KGaA, Darmstadt, Germany). The following reagents were sourced from Sigma-Aldrich (Johannesburg, South Africa): hydrochloric acid (37%), reagent grade; ethanol (96.8%); Invertase from baker's yeast (*S. cerevisiae*), Grade VII, 300 U/mg solid; D-(+)-Glucose, ACS reagent; copper (II) chloride, powder, 99%; neocuproine (anhydrous 2,9-dimethyl-1,10-phenanthroline), ≥98%; absolute ethanol; sodium hydroxide, reagent grade, ≥98%; sodium carbonate, ACS reagent, ≥99.5%; potassium sodium tartrate tetrahydrate, ACS reagent, ≥99%; ammonium sulfate, ACS reagent, ≥99%; methyl cellulose, M0387, viscosity 1,500 cP; epicatechin, analytical standard; Folin-Ciocalteu phenol reagent; gallic acid monohydrate, ACS reagent, ≥98%; Bradford reagent; bovine serum albumin, heat shock fraction, pH 7, ≥98%; sulphuric acid, ACS reagent, 95–98%; liquified phenol, ≥89%; D-(+)-Mannose, analytical standard; dextran from *Leuconostoc mesenteroides*, analytical standard for GPC, Mw: 5k, 25k, 50k, 150k, 410k, 670k; phosphate-buffered saline, tablet; 3,3′,5,5′-tetramethylbenzidine, ≥99%; potassium citrate, ≥98%; hydrogen peroxide, 30% (w/w) stabilized; tetrabutylammonium borohydride, 98%; N,N-dimethylacetamide, anhydrous, 99.8%;

Monoclonal antibodies INRA-RU1, INRA-RU2, were obtained from INRAE, France; BS-400-2, BS-400-3 from Biosupplies Australia, Victoria; LM2, LM5, LM6, LM7, LM11, LM15, LM16, LM19, LM20, LM21, LM23, LM24, LM25, JIM5, JIM7, JIM8, JIM13, JIM15, JIM16 from Kerafast, Inc, Boston, MA, USA. A monoclonal anti-RGII antibody was the kind gift of Professor Masaru Kobayashi (Kyoto University, Japan) (Zhou, Kobayashi, Awano, Matoh, & Takabe, 2018). In addition, secondary antibodies, all HRP-conjugated, were purchased from Biocom Africa (Pty) Ltd, Centurion, South Africa, including goat anti-rat IgM heavy chain antibody, goat anti-rat IgG heavy and light chain antibody, goat anti-rat IgA heavy chain antibody, donkey anti-mouse IgG heavy and light chain antibody, goat anti-rat IgG2a antibody, goat anti-rat IgG2c antibody.

2.2. Wine

Sixteen red wines made from Cabernet Sauvignon grapes (harvest years 2018, 2019, and 2020) were selected for analysis (see Kuhlman et al. (2022) for vineyard and vinification details of the research wines). Of the selected sixteen, nine were made with grapes from the same research vineyard, and seven were samples from commercial wineries in the Stellenbosch area. All wines were fermented by inoculation at crush with 20 g/hL Lalvin ICV D21 yeast (Lallemand Oenology, Ontario, Canada) as instructed by the manufacturer. All wines were fermented to dryness (<0.5 g/L residual sugar), pressed, and allowed to complete malolactic fermentation with Lalvin VP41™ *Oenococcus oeni* preparation (Lallemand Oenology, Ontario, Canada), as per manufacturer instructions. Eight wines were produced using commercial preparations of pectolytic enzymes (four research wines and four from commercial wineries). The commercial pectolytic enzyme used was 5 g/hL Lafase® HE Grand Cru enzyme preparation (Laffort, Bordeaux, France) per manufacturer instructions. Lafase® HE Grand Cru contains predominantly polygalacturonase activities with minor side activities (see Gao et al., 2016; Romero-Cascales, Ros-García, López-Roca, & Gómez-Plaza, 2012). Eight wines were made without exogenous enzymes (five research and three commercial). All wines were stored in the bottle at 15 °C for at least six months before analysis.

2.3. Polysaccharide precipitation

Loosely following the work of Ayestarán et al. (2004), 1.5 L of each wine sample was reduced to 300 mL using a rotary vacuum still

(Rotavapor R-100, Büchi Labortechnik AG, Falwil, Switzerland) at a water bath temperature of 40 °C and a coolant temperature of 1.0 °C. This concentrate was added to 1700 mL ice-cold 25 mM HCl in 95% ethanol (aq). This mixture was left for 12 h at 4 °C, and then the precipitate was filtered with #100 Waterman filter paper. The filtrate was scraped from the filter paper into 50 mL centrifuge tubes and washed with 45–50 mL 25 mM HCl in 80% ethanol (aq) at 4 °C for 30 min under agitation. The sample was centrifuged for 10 min at 4000 g at 4 °C, and then the supernatant was discarded. The pellet was resuspended in the 80% acidified ethanol as above, and the process was repeated until the supernatant was clear and colourless, ensuring the elimination of anthocyanins. The washed pellet was dried in the open centrifuge tube at 40 °C overnight and then suspended in 5 mL ultra-purified water, frozen at −80 °C, and lyophilised (Christ Lyophilizer, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) until completely dry.

2.4. Free reducing sugar assay

Polysaccharide extractions from each of the 16 wines were tested for simple sugars using a modified assay from Başkan, Tütem, Akyüz, Özen, and Apak (2016). Ten milligrams of dried precipitate was dissolved in 1 mL ultra-purified water and passed through a Supelco Discovery DPA-6S SPE cartridge (Sigma-Aldrich, Johannesburg, South Africa). The cartridge was rinsed with 2 mL ultra-purified water, which was combined with the sample eluent. One hundred microlitres of the sample eluent was combined with 1900 μL of 1000 U/mL invertase enzyme in ultra-purified water and held for 20 min at 55 °C in a water bath to convert sucrose to fructose and glucose. Two hundred and 50 μL of the sample was combined with 250 μL of the following reagents: 10 mM $CuCl_2$ (aq), 15 mM neocuproine in absolute ethanol, 500 mM NaOH (aq) with 2% (w/v) Na_2CO_3, and 100 mM sodium potassium tartrate (aq). This mixture was held at 60 °C for 20 min, and then 300 μL was transferred to a 96-well acrylic spectrophotometer plate, and absorbance was read at 485 nm on a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). This same instrument was used for all further spectrophotometric analyses. A standard curve was prepared using eight glucose concentrations from 1 to 0.00625 mM; all samples were tested in triplicate. No precipitate samples had any detected reducing sugars (limit of detection 0.03 mM glucose equivalent, data not shown).

2.5. Methycellulose precipitation assay for tannins

Polysaccharide extractions were analysed for tannins using a methylcellulose method modified from Sarneckis et al. (2006). Each sample was dissolved at 10 mg/mL in ultra-purified water. Twenty-five microlitres of the extraction was added to 200 μL of a saturated aqueous ammonium sulfate solution and 700 μL of a 0.4% aqueous methylcellulose solution. The mixture was vortexed and allowed to sit at room temperature for 10 min before being centrifuged at 4000 g for 10 min. The supernatant was transferred to 96-well plates (Greiner UV Star 370, Grenier GmbH, Kremsmünster, Austria). The absorbance at 280 nm was measured on the spectrophotometer. A blank was prepared using ultra-purified water in place of the polysaccharide solution. A standard curve was prepared using epicatechin; all samples were prepared in triplicate. All polysaccharide extractions had responses below the limit of detection (7.5 mg/L epicatechin equivalents).

2.6. Total phenols using the Folin-Ciocalteu assay

Total phenols were analysed using the Folin-Ciocalteu method modified for 96-well plates (Attard, 2013). Each polysaccharide extract was dissolved at 10 mg/mL in ultra-purified water for analysis. In a 96-well acrylic spectrophotometer plate, 50 μL of the sample was combined with 12.5 μL of Folin-Ciocalteu reagent. The plate was shaken for 30 s using the plate shaker function of the spectrometer. One hundred and 63 μL 7.5% (w/v) Na_2CO_3 (aq) was added to each well, and the plate was shaken for 30 s. The plate was floated on a 40 °C water bath for 30 min, and then the absorbance was measured at 725 nm. A standard curve was prepared using ten concentrations of gallic acid from 1 g/L to 0.005 g/L; all samples were analysed in triplicate. All precipitates analysed had total phenols below the limit of quantification (0.03 mg/L gallic acid equivalent).

2.7. Protein content using the Bradford assay

Finally, all polysaccharide precipitates were analysed for protein content using the Bradford assay (Stoscheck, 1990). All precipitates were dissolved at 1 mg/mL in ultra-purified water. One hundred and 60 μL of the sample was combined with 40 μL of Bradford reagent in a 96-well acrylic spectrophotometer plate and were mixed well with a pipette tip. The samples were incubated at room temperature for 5 min, and then absorbance at 595 nm was measured using the spectrophotometer. A standard curve was prepared using bovine serum albumin in six concentrations from 50 to 1.563 mg/L. Polysaccharide precipitates had a mean protein content of 1.986% (dry weight basis, SD 0.25%). While wines produced with enzymes had a slightly higher protein concentration (2.014%) than non-enzyme macerated wines (1.957%), the difference was not statistically significant (p-value = 0.4240).

2.8. Sulphuric acid phenol carbohydrate assay

A colourimetric method described by Masuko et al. (2005) was used with slight modifications. In brief, 50 μL of the sample was added to a 96-well polypropylene spectrophotometer plate (Grenier GmbH, Kremsmünster, Austria). One hundred and 50 μL of concentrated sulphuric acid was added to the sample in the well, followed by 30 μL of 5% liquid phenol in ultra-purified water. The tray was floated, uncovered, on a 95 °C water bath for 5 min and then transferred to a room temperature water bath for 5 min. Absorbance was read at 490 nm using the spectrophotometer. All analyses were performed in triplicate. A standard curve was created using mannose at 2, 4, 8, 16, 32, 64, and 128 nmol per well. To measure total carbohydrates in wine, 500 μL of wine was passed through Supelco Discovery DPA-6S SPE cartridges to remove phenolic compounds before acid hydrolysis.

2.9. Gel permeation chromatography

All high-pressure liquid chromatography was performed on an Agilent 1100 with a G1362 diode array detector and a G1362A refractive index detector connected in series. As outlined by Sathitnaitham et al. (2021), ultra-purified, degassed water was filtered through a 0.22 μm Millipore filter and used as the eluent at 0.5 mL/min. A Cytiva Superose 6 Increase 10/300 GL column (Sigma-Aldrich, Johannesburg, South Africa) was used for separation. The refractive index was captured, as well as absorbance at 192, 204 and 280 nm. Initial work established that for wine polysaccharide precipitate, there was no signal after approximately 47 min, so the run time was fixed at 50 min (data not shown). Samples were prepared at 10 mg/mL in 5% (v/v) ethanol and filtered through 0.22 μm nylon syringe filters before being injected at 100 μL per injection unless otherwise specified.

2.10. Molecular weight separation

Six dextran molecular weight standards of 5, 25, 50, 150, 410, and 670 kDa were used to build a model of molecular size by retention time. Dextran molecular weight standards were dissolved at 10 mg/mL in ultra-purified water and filtered as above. Upon injection in triplicate, peaks were identified at 37.6, 33.2, 30.2, 24.8, 21.4, 19.9 min for 5, 25, 50, 160, 410 and 670 kDa standards, respectively. An exponential curve was fit with an R^2 value of 0.9932: $y = 116877\ e^{\wedge} -0.262x$. According to

the manufacturer's specification, this column separates molecules in the range of 5 to 5,000 kDa, correlating to an effective separation window of 14.3–37.6 min. Dextrans are linear glycans and when comparing hyperbranched colloids it is possible we are slightly overestimating the wine colloids molecular weight using this approach.

2.11. Mannoprotein separation

A Cytiva Hi-Trap Con-A 4B (5 mL) column (Sigma-Aldrich, Johannesburg, South Africa) was used to remove mannoproteins from each polysaccharide extract. The column was prepared by flushing with 5 column volumes (CV) of binding buffer at 5 mL/min: 20 mM Tris-HCl, 500 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, adjusted to pH 7.4. The column was then flushed with 5 CVs of ultra-purified water at 5 mL/min. One millilitre of each sample was prepared at 10 mg/mL and injected at 1 mL/min, followed by 5 mL of ultra-purified water at the same flow rate. Next, 25 mL of ultra-purified water was passed at 4 mL/min and captured. Finally, 5 mL more ultra-purified water was passed at the same flow rate to flush the column; this eluent was discarded. After nine samples (calculated at less than half of the capacity of the column according to manufacturer's specifications), the column was flushed with 25 mL of 20% ethanol in ultra-purified water to remove captured mannoproteins and then re-equilibrated with 25 mL of binding buffer and 25 mL of ultra-purified water before proceeding with the subsequent nine samples.

2.12. Fraction collection

A Waters Fraction Collector III (Waters Corporation, Milford, MA, USA) was used in blind tandem with the Agilent 1100 HPLC system. Four 96-well polypropylene spectrophotometer plates (Grenier GmbH, Kremsmünster, Austria) were mounted, collecting seventy-two 0.25 mL (30 s of run time) fractions from RT 10–46 min, allowing collection of 5 fractionated samples in 4 plates. During fraction collection, the fraction collector was cleaned using 80% ethanol and enclosed with an antibacterial food film (Hitachi Chemical Co., Ltd, Toyko, Japan). Plates were sealed with silicon plate covers and immediately stored at −20 °C.

2.13. ELISA identification of cell wall polysaccharide epitopes

Polysaccharide epitopes were identified using an ELISA based on horseradish peroxidase labelled antibodies (Supplementary Table 1), using a method developed from the work of Pattathil et al. (2010) and Sathitnaitham et al. (2021).

For analysis, 50 μL of the sample was transferred into 96-well flat-bottom plates treated for tissue culture (Costar 3599; Corning, New York, USA) and dried at 37 °C overnight. The plates were blocked with 3% bovine serum albumin (BSA) dissolved in phosphate-buffered saline (PBS) for 1 h at 37 °C, covered. The wells were emptied and probed with 25 μL of primary antibody diluted 1:50 in 1% BSA in PBS for 1 h at 37 °C, covered. The plates were washed three times with PBS prepared at 10% strength in distilled water. The secondary antibody was diluted 1:10,000 in 1% BSA in PBS and added to the wells at 50 μL for 1 h at 37 °C, covered. Finally, the plates were washed six times with 10% PBS in distilled water.

A 3,3′,5,5′-tetramethylbenzidine (TMB) system developed by Frey, Meckelein, Externest, and Schmidt (2000) was used with a modified TMB concentration of 0.1 g/L or 0.416 mM in the TMB solution. Seventy-five μL of TMB solution was added to each well, and the plates were incubated at room temperature for 30 min. One hundred and twenty-five μL of 1M sulphuric acid (aq) was added to stop the colour development. Plates were measured for absorbance at 450 nm on the spectrophotometer. All measurements were made in triplicate.

Whole polysaccharide precipitates were screened against all antibodies at a concentration of 0.1 mg/mL. All spectrophotometer readings were selected against a 3:1 signal-to-noise ratio and then normalized. After the screening, nine antibodies showed significant signals in the 16 polysaccharide precipitates: INRA-RU2, JIM7, LM15, JIM8, LM16, LM25, JIM13, JIM16, and LM2. A second screening at 1.5 μg/mL polysaccharide extract eliminated JIM15 as a probe due to low response. Molecular weight fractions were diluted 20 times before analysis and tested against the eight chosen epitope probes.

2.14. Statistical analysis

One-way or two-way ANOVA was used as indicated for multivariate analysis, followed by post hoc testing using Tukey's HSD. Other data were compared using Student's t-test or Welch's *t*-test for sample sets with equal or unequal variance, respectively. One-tailed p-values were calculated for experimental situations where no negative results were possible, otherwise, a two-tailed p-value was used. All calculations and visualisations were processed using Python 3.9 software; curve fitting and statistical analysis was performed using bioinfokit c

3. Results and discussion

3.1. Total carbohydrate content between control and enzyme wines

All wines were analysed using the sulphuric acid–phenol analysis for the total carbohydrate content, and the results are presented in Table 1. Sampled wines thus had concentrations of carbohydrates from 409 to 1724 mg/L. This generally agrees with the total carbohydrate concentration in wine made from Cabernet Sauvignon and other red varietals (Ayestarán et al., 2004; Gil et al., 2012; Guadalupe & Ayestarán, 2007). However, Ayestarán et al. (2004) found that enzyme-maceration increased the concentration of polysaccharides in wine, and Doco et al. (2007) found that it decreased polysaccharide concentration. When considering the use of enzymes in wine production in the present, there is no significant difference between enzyme treated and untreated wines in relation to their concentration of carbohydrates (Table 1). The remarkably high concentration of wine 12 at almost 5000 mg/L appears anomalous and we do not have a clear explanation for this. On average we see wine polysaccharide content to be in the range 400–1800 mg/L. That is, we do not see a consistent pattern in the datasets. This agrees with previous work that found similar results based on acid hydrolysis and gas chromatography analysis to determine relative polysaccharide concentrations (Kuhlman et al., 2022) where no clear trend was observed. Romero-Cascales et al., 2012; Gao et al., 2016 characterized Lafase® HE Grand Cru and found it predominantly contained polygalacturonase activity with minor side activites.

Table 1
Total carbohydrate concentration of the Cabernet Sauvignon wines measured using the phenol sulphuric acid assay.

Sample	Harvest year	Treatment	Concentration (mg/L)	pH	Alcohol %
w1	2018	Control	1724 ± 282	3,31	11,9
w2	2018	Enzyme	1239 ± 517	3,57	12,0
w3	2018	Control	1131 ± 416	3,39	12,4
w4	2018	Enzyme	1514 ± 245	3,43	12,6
w5	2019	Control	838 ± 43	3,42	12,1
w6	2019	Enzyme	460 ± 64	3,30	12,3
w7	2019	Control	409 ± 80	3,25	12,9
w8	2019	Enzyme	1015 ± 498	3,47	13,0
w9	2018	Enzyme	676 ± 74	3,26	14,1
w10	2018	Enzyme	638 ± 91	3,64	14,7
w11	2019	Enzyme	694 ± 39	3,66	14,4
w12	2020	Enzyme	4979 ± 231	3,57	14,5
w13	2020	Control	847 ± 359	3,47	14,2
w14	2020	Control	780 ± 613	3,48	14,4
w15	2019	Control	991 ± 64	3,41	13,9
w16	2019	Control	431 ± 14	3,44	12,9

3.2. Overall polysaccharide size distribution between control and enzyme wines

Each polysaccharide extract was analysed using gel permeation chromatography. Using the refractive index, the concentration of polysaccharides in the solution at any point in the separation can be calculated (Cheong, Wu, Zhao, & Li, 2015; Wu et al., 2016). Individual measurements can be combined across the entire chromatogram to give a cumulative total. Maintaining injection concentration ensured there was very little shift in separation by retention time. Dextran standards from 5 kDa to 670 kDa demonstrated a reliable correlation between retention time and molecular weight of the standard. A clear peak is seen where the ethanol exits the column at a retention time of about 43 min, which correlates to a molecular weight of around 1000 kDa. Taking only compounds that elute at 5 kDa and higher, a repeatable separation is obtained. The highest range of separation for this column, according to the manufacturer, is 5000 kDa.

Differences between the molecular weight profiles of the wines can be detected in the 85–105 and 1000–6000 kDa regions (Fig. 1). When grouped by harvest year, harvest 2019 wines had more high molecular weight polysaccharides than the 2018 or 2020 wines. Contrasting different treatments (bottom graph of Fig. 1) shows two areas of interest: 85–105 kDa and 1050–6000 kDa. Enzyme-treated wines have a lower amount of polysaccharides in both weight ranges. However, three samples showed quite variable profiles for which we do not have a clear reason. To properly contrast these differences, the influence of yeast-derived mannoproteins must be considered.

3.3. Excluding yeast mannoproteins and mannose-containing polysaccharides from the grape-derived polysaccharides in the wines

Mannoproteins compose a significant part of the total polysaccharides found in wine, as much as 50% reported by some authors (Guadalupe et al., 2007; Vidal et al., 2003). Produced during fermentation and released when yeast cells lyse, these mannoproteins are often studied in tandem with plant cell walls in wine systems as they are difficult to separate. This study uses a concanavalin-A linked resin to separate these two polysaccharide classes to see what difference might be obscured by their usual co-presence.

Different molecular weight profiles between the whole precipitates, mannoprotein-separated precipitates, and mannoproteins can be seen in the operational range of the column (Fig. 2). There is a grouping of mannoproteins around 110–130 kDa, but the variation between samples in that part of the size distribution is quite large, so a statistically significant relationship cannot be shown for this band (p-value = 0.1831). Other researchers have also seen mannoproteins in the 100–300 kDa range (Poncet-Legrand, Doco, Williams, & Vernhet, 2007; Saulnier, Mercereau, & Vezinhet, 1991). The region from 1000 to 5000 kDa shows a marked difference (p-value <0.05). This size range for mannoproteins is larger than most other workers report (Guadalupe et al., 2015; Kassara, Li, Smith, Blando, & Bindon, 2019), but is echoed by size-distributions of whole polysaccharide extracts from wine as determined by Guadalupe et al. (2007).

When taken on average, treatment with concanavalin-A resin removed about 15.3% of the total polysaccharides present in the extract.

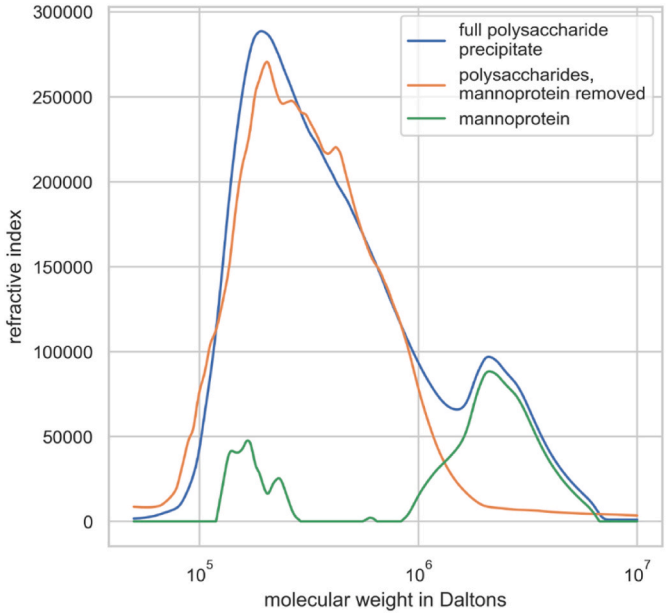

Fig. 2. Mannoprotein fraction of whole polysaccharide extract shown along with the full polysaccharide precipitate and the same precipitate after concanavalin A affinity chromatography treatment.

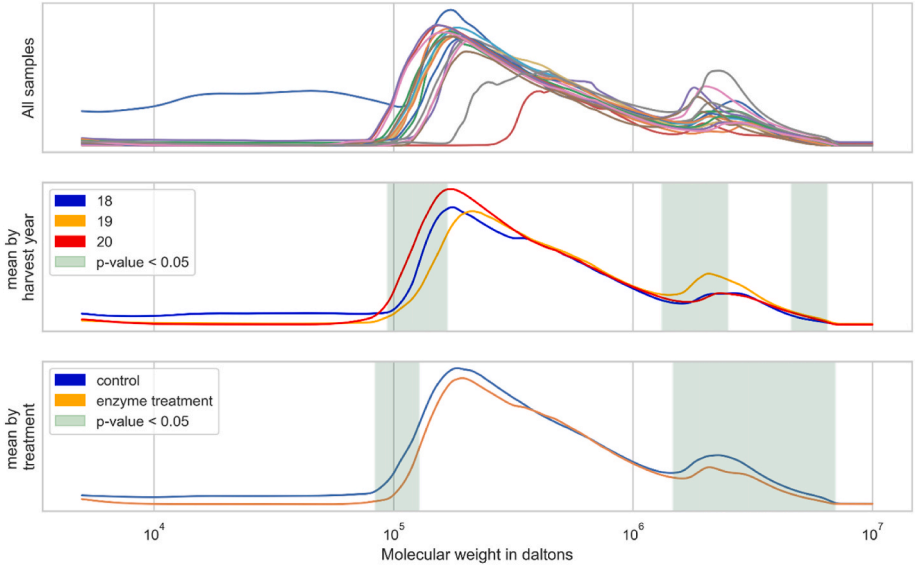

Fig. 1. Polysaccharide molecular weight distribution by sample, year, and treatment of the Cabernet Sauvignon wines tested. Y-axis is normalized to show the comparative value between each graph. Green bands indicate areas where the p-value <0.05.

When also including the less statistically significant double (or possibly triple) peak found between 110 and 300 kDa, the total mannoprotein percentage rises to 19.6%. These low molecular weight fractions could represent degraded mannoproteins. This is on the lower end of the range reported by others but mirrors the findings of Vidal et al. (2003).

When yeast-derived mannoproteins are excluded, a comparison of treated and non-treated wine polysaccharide molecular weight distributions produces four main areas of interest, sub 50 kDa, the 85–150 kDa band, the 190–400 kDa band, and the 1000+ kDa band (Fig. 3) (see Ducasse et al., 2010, 2011). There appears to be a clear reduction in polymer size in enzyme-treated wines reflected in the loss of signal in the 190–400 kDa band, and the increased signal in the 85–150 kDa band (see Ducasse et al., 2010, 2011). Previous work has established molecular weight ranges for several polysaccharide categories in red wine, including arabinogalactan proteins, mannoproteins, and rhamnogalacturonans (see Ducasse et al., 2010, 2011). Arabinogalactan proteins have been reported with a wide range of sizes between 20 and 800 kDa (Doco, Vuchot, Cheynier, & Moutounet, 2003; Pellerin, Vidal, Williams, & Brillouet, 1995; Vidal et al., 2003). Rhamnogalacturonan-I purified from wine were reported with molecular weights from 44 to 52 kDa by Vidal et al. (2003).

Focusing first on the blue trace in Fig. 3, one sees a relatively normal peak shape with indicators of co-eluting polymers at 190 and 250 kDa. AGPs have been previously reported with similar molecular weights, but it is notable that there are no visible peaks that might represent the other two classes of plant wall polysaccharides expected in red wine. RG-II would be expected at 12 kDa (and possibly 5–6 kDa for monomers), and RG-I at 44–52 kDa.

Comparing the blue and orange trace in Fig. 3, Enzyme use reduces the 190–400 kDa band by about 17.2% of the total control polysaccharide fraction, which might indicate a reduction in larger-sized AGPs and arabinogalactans. There are also differences above 1050 kDa, a loss of about 10.6% when taken together with sub 9 kDa polymers. It is also notable that in the region of 400–1050 kDa, there is minimal effect of Lafase® HE Grand Cru enzyme treatment on size distribution. On the enzyme chromatogram, a suggestion of a peak at 650 kDa, when taken with the peak at 450 kDa indicate that these likely AGPs are not affected by the specific pectolytic enzymes used in this study (see Gao et al., 2016). Two peaks that are found on the control chromatogram at 190 and 250 kDa appear to shift as the entire enzyme curve widens as the peak heights drop, lowering the mean molecular weight.

A notable observation (or lack of observation) needs to be discussed at this point. Rhamnogalacturonan-II polysaccharides are reported to be between 15 and 19% of total red wine polysaccharides (Ayestarán et al., 2004; Martinez-Lapuente et al., 2016; Vidal et al., 2003). Rhamnogalacturonan type-II dimers have been reported with an average molecular weight of 10–12 kDa (Kobayashi, Matoh, & Azuma, 1996; Pellerin et al., 1996). One would expect to see a significant refractive index detector signal in the 10–12 kDa region, but this work consistently does not see any indication of a grouping of polysaccharides of that size. The size separation limit of the column is reported at around 5 kDa, so it could be possible that small oligosaccharide separation is not reliable and that the RG-II polysaccharides are being eluted with the ethanol peak at the end of the separation. Monomers although possible are highly unlikely to be present in red wine, determined to be approximately 4.7 kDa by the preceding work, which could be small enough to elude separation. It is also possible that the vines could be suffering from a boron deficiency and both monomeric and dimeric RGII are present in the macerated grapes during winemaking. As most RG-II in planta is thought to be dimerized (as is required for plant growth) and the kinetics of a monomerization in a wine system is not well understood (Chormova, Messenger, & Fry, 2014), it is not clear that any significant portion of the dimers would separate. A recent work separating RG-II from both plant extracts and red wine had success with a similar column (Superdex 75 Increase 10/300 GL) to the column used here (Superose 6 Increase 10/300 GL); however, it was sized for molecules between 3 and 70 kDa, a much smaller range with a lower separation limit than the Superose 6 Increase (5–5000 kDa). The authors also reported a failure to separate RG-II using Sephadex 75 resin, which has a separation range from 4 to 50 kDa (Barnes et al., 2021). It is possible that RG-II was too small to separate reliably or that the specific conformation of RG-II made it elute in a smaller molecular weight range than expected on the gel resin selected. There is also the possibility that lack of sufficient ionic strength could have caused the RG-II to remain on the column system. We also screened our fractions using a recently developed monoclonal antibody against RGII (a kind gift Professor Masaru Kobayashi (Kyoto University, Japan) (Zhou et al., 2018)) but achieved no positive signals with this probe (data not shown). We did however try two concentrations during method development (0.05M and 0.1M $NaNO_3$) which didn't yield any more separation in the lower molecular weight ranges, nor did it shift the size groupings seen using dH_2O. No extra peaks indicating that low molecular weight polysaccharides were being retained on the column were seen at these relatively weak ionic concentrations. Currently we do not have a clear explanation for these observations.

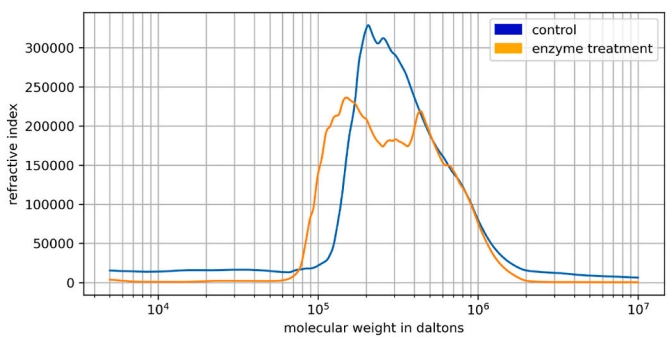

Fig. 3. Summary of control and enzyme-treated wine polysaccharide precipitates, mannoprotein excluded (all wines). N = 7 control wines, N = 8 enzyme-treated wines.

3.4. ELISA epitope identification of the separated high molecular weight wine polysaccharides

Immunodetection is a powerful technique used to identify structurally unique molecules, most commonly proteins. Its use expands to other compound classes, including polysaccharides and glycoproteins; however, it does have unique limitations in use with polymers. Monoclonal antibodies (mAbs) are created by challenging hybridoma cells of a specific host with a purified standard of the desired target molecule. Because the antibody creation is unsupervised (or supervised by the cellular machinery of the host system and not directly controlled by researchers), the specific region of interaction between an antibody and the target molecule is not predictable. A cross-reactive antibody can interact with a similar or identical region of interaction on a different molecule, yielding a false positive. For many polymer systems, such as polysaccharides, the number of base molecules and the possible linkages between them are fewer than for most proteins (Bordeaux et al., 2010). The folded architecture of proteins is often more varied than polysaccharide systems (Bordeaux et al., 2010). Fewer building blocks and a less complicated architecture yield a mathematically reduced number of unique binding sites (Bordeaux et al., 2010). In polysaccharide epitope immunochemistry, it is accepted that many monoclonal antibodies will be cross-reactive with structurally similar polymers, but the exact stoichiometric ratio of those interactions is not possible to predict, leading to a less certain interpretation of immunochemistry analysis (Bordeaux et al., 2010). To wit, if monoclonal antibody A binds to four spots on homogalacturonan A while monoclonal antibody B only binds to one spot on homogalacturonan B, it would appear there are four times the amount of homogalacturonan A if the data is analysed quantitatively

(Bordeaux et al., 2010). Instead, the analysis performed in this work compares the mAbs signal across samples, but not directly to other monoclonal antibody signals, relying on previously discussed refractive index detection for quantification by molecular size.

3.5. Screening of whole polysaccharide precipitates against cell wall probes using ELISA

Whole polysaccharide precipitates were screened against 24 monoclonal antibodies (after mannoprotein exclusion) (Fig. 4; full list of antibodies in Supplementary Table 1). Values are normalized globally to a maximum signal of 100, with a minimum threshold of five. From the individual samples, one can see only PPT 8 gives a low signal for LM6 (arabinans) and LM18 (homogalacturonans), only PPT 1 has a low signal for LM7 (homogalacturonans). In contrast, LM5 (glucans), JIM5 (galactans), BS400-2 (glucans), BS400-3 (glucans), LM 19 (homogalacturonans), INRA-RU1 (rhamnogalacturonan-I), and LM24 (xyloglucan) have no signal at all. Strong signals are seen for several arabinogalactan protein probes as well as RG-I and one xyloglucan probe. This initial screening was performed at a very high concentration—higher than the actual analysis concentration window for ELISA, meaning several of the probes reached the maximum signal of the assay. Eight probes from the first screening had signals that were quantifiable (at least 10 times the signal to noise ratio): JIM13, JIM8, LM2, JIM7, LM16, RU2, LM15, and LM25. These eight were selected for further exploration. An optimal concentration for ELSIA was determined to be 1.5 μg/mL, a thousand times more diluted than the initial screening, which allowed differentiation between the most probes while not exceeding the limits of the assay (data not shown).

3.6. Epitope mass profiling of enzyme and control wine polysaccharides

Using the refractive index chromatograms from each sample, fractions covering 3 kDa–1050 kDa were tested against the 8 previously identified epitope probes using ELISA. For all fractions, JIM13, JIM8, LM16, LM2, and LM25 significant signals were detected, while JIM7, LM15, and RU2 did not (data not shown). Data from significant signals was grouped by monoclonal antibody epitope target and normalized globally, and then the signals that target the same epitope were averaged to give a unified epitope signal (Fig. 5).

When compared to the size distribution as shown by the RID signal, the control polysaccharide signals show the highest signals for rhamnogalacturonan-I (RG-I, probe LM16), xyloglucans (XyG, probe LM25) and arabinogalactan proteins (AGP, probes LM2, JIM8, JIM13) between 200 and 1100 kDa. The probe signals follow a relatively standard distribution. For xyloglucans, there is a small but detectible signal from the smallest mW polysaccharides until about 150 kDa, from where an increase is observed to a peak signal around 450 kDa. After that, the

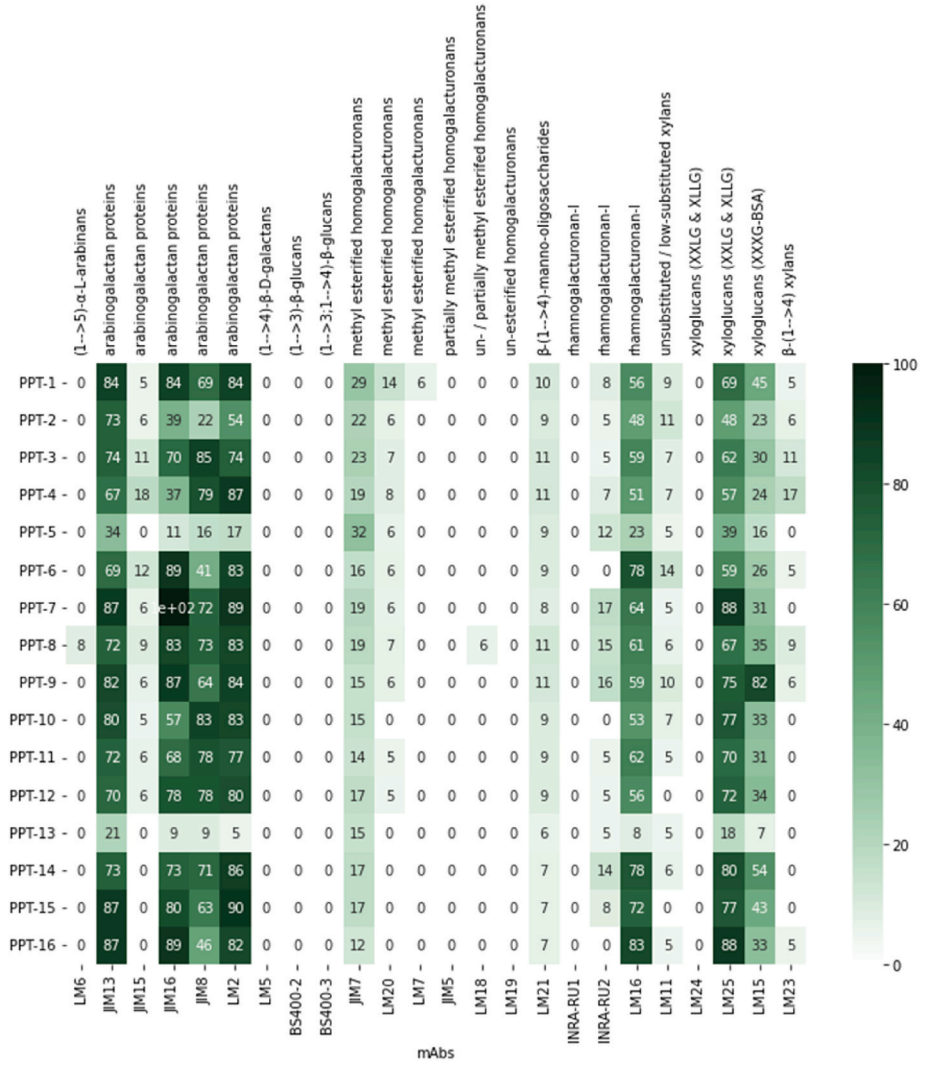

Fig. 4. Screen of all wine polysaccharide precipitates (PPT) against plant probes. The values are normalized globally with a low-signal cut-off of 5. The bottom of the x-axis indicates the specific probe, and the top of the x-axis is labelled per target epitope.

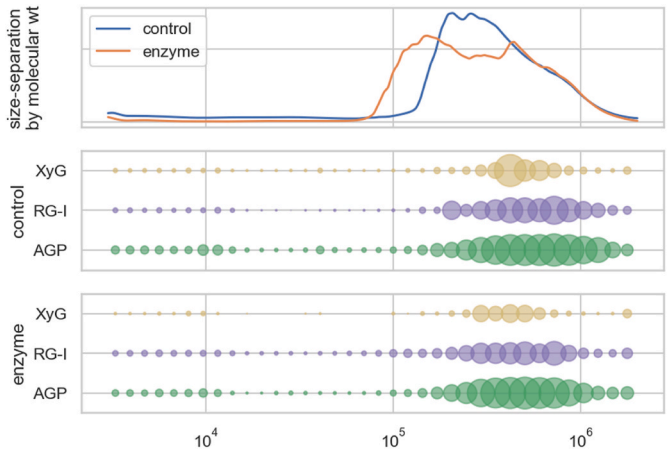

Fig. 5. Epitope probe signal by treatment, compared with the refractive index signal from averaged chromatograms for each treatment. X-axis in Daltons, specific epitope class indicated in the y-axis, each circle is a measurement, sized in relation to the corresponding ELISA signal from 0 to 100. XyG: LM25, RG-I: LM16, AGPs: LM2, JIM8, JIM13 Signals lower than 5 do not appear at all.

signal steadily decreases as the molecular size increases. There is a small increase mirrored by the AGP signal at about 30 kDa. Rhamnogalacturonan-I epitopes follow a similar pattern, but the range of high signal is broader, stretching from around 200 to 1050 kDa with a signal peak at 500 kDa. RG-I exhibits a small but consistent signal from 5 to 10 kDa, weakening significantly from 10 kDa to the start of the peak at about 150 kDa. AGPs give the highest signal out of all the epitopes and have a similar pattern to RG-I; the response max is found at 600 kDa, with an even distribution from 100 kDa to 1100 kDa. Sub 10 kDa AGP polymers also show an increased signal compared to the baseline.

Comparing the untreated wines with enzyme-macerated wines, we see both a reduction in maximum XyG intensity and a shift from 400 kDa to closer to 300 kDa, with reduced signal intensity from every molecular weight fraction. XyG is not detected in the 10–12 and 40–100 kDa band. The 5–10 kDa group shows a reduction in signal. RG-I and AGPs have similar responses in enzyme-treated wine for larger polymers; there is a loss of signal, and the centre of the peaks shifts to the left, indicating similar distribution but smaller mean size of polymers by some 100 kDa or more. RG-I has increasing prevalence of polymers in the sub 8 kDa and 10–130 kDa range, compared to non-treated samples. AGP has the least signal loss in comparison between treatments, but there is a definite decrease in the highest molecular weight polymers, causing the peak to shift 100 kDa lower.

The detection of xyloglucans in wine polysaccharides is unexpected, but has been reported by Gao, Fangel, Willats, Vivier, and Moore (2015) in concentrated wine polysaccharides using a comprehensive microarray polymer profiling method. While xyloglucans are a known structural element of the plant cell wall and are often detected in grape berries, they are most often extracted from tissues under alkaline conditions, not a native state for any part of the winemaking process. It is also curious that AGP epitopes would be affected by a Lafase® HE Grand Cru as they lack the enzyme-specific target site for cleavage. While RG-I (and RG-II) are both part of the pectic family of galacturonans, arabinogalactan proteins are not. Strong immunochemistry signals for RG-I, XyG and AGP together in polymers sized between 300 and 800 kDa provides evidence that this grouping of polysaccharides is primarily composed of AGP-linked RG-I epitopes bonded to xyloglucan. The existence of two separate xyloglucan molecular weight groupings was reported by Talbott and Pickard (1994); monosaccharide analysis showed that the larger polymers (the 400–6000 kDa region) had monosaccharide ratios that correlated with a mixed arabinogalactan-xyloglucan polymer. In a separate investigation (see Popper & Fry, 2008), evidence for a mechanism that covalently bonded xyloglucan and RG-I epitopes was reported; the authors also noted that up to 50% of the xyloglucan synthesised was built on an anionic primer, most likely RG-I, which yielded an negatively charged xyloglucan-pectin complex with a negative net charge. Others have identified an "arabinoxylan pectin arabinogalactan protein" in which AGP was linked to RG-I via a rhamnosyl residue, and arabinoxylan to RG-I via either the rhamnosly residue in the RG-I domain or an arabinosly domain from the AG glycan domain (Tan et al., 2013). Therefore, arabinogalactan proteins could be linked to xyloglucan polymers via a rhamnogalacturonan I bridge yielding a co-polymer as suggested from the data presented. The implications of this particular pectinase-resistant AGP-rich co-polymer is yet unclear and may be significant in impeding complete maceration of grapes during ferment and wine colour extraction impacting sensory characteristics in red wine.

Enzyme-maceration reduced the signal of both RG-I, which was expected as pectolytic enzymes specifically target the homogalacturonan domain it contains, but also xyloglucan and more moderately, AGPs, providing further evidence that arabinogalactans and xyloglucans are linked by rhamnogalacturonan-I in the cell wall. However, NMR analysis of the purified polymer as done by Tan et al. (2013) would be useful to pursue. The RG-I backbone of repeating rhamnose/galacturonic acid has been shown to be susceptible to lysis by some commercial preparations of pectolytic enzymes (Ducasse et al., 2011), and many of the side chains that form the RG-I structure are homogalacturonans, with vulnerable 1,4-α linkages (Kaczmarska, Pieczywek, Cybulska, & Zdunek, 2022; Romero-Cascales, Fernández-Fernández, Ros-García, López-Roca, & Gómez-Plaza, 2008; Romero-Cascales et al., 2012). The limited cleavage of RG-I causes a reduction, but not complete destruction, of this polymer even though the enzyme dose is well in excess and sufficient ferment time had elapsed. This limited cleavage, in lieu of a more complete disassembly, might be due to the conformation of the co-polymer, which could block access to the cleavage site, causing a masking effect in large molecular weight polysaccharides.

4. Conclusions

This work explored the variation in polysaccharide content and composition in red wines. Total polysaccharide content was mostly in the range of 400–1800 mg/L, and the concentration of polysaccharides was not correlated with enzyme use in any systematic way.

Yeast-derived mannoproteins and polysaccharides containing mannose were found to have two size ranges: 100–250 kDa and 850–5000+ kDa. Upon removal with affinity chromatography, grape-derived polysaccharides were detected in the 85–1100 kDa range. Enzyme-assisted maceration was correlated with a major effect on polysaccharides with molecular weight 190–450 kDa, and a lesser effect on the range 85–150 kDa. By expanding the sample size and varietals considered, future research could work towards a comprehensive model utilizing these specific changes in size profile to detect enzyme usage in an unknown wine. Specific epitope detection by immunochemistry also showed a quantifiable difference between treatments. The concentration of XyG, AGP, and RG-I appear to concentrate at higher molecular weights (300–1050 kDa); this result was surprising, as a more size-varied, multi-modal distribution had been expected. It is tentatively suggested that this grouping is an arabinogalactan protein covalently linked to rhamnogalacturonan-I while it is covalently linked to xyloglucan. We however only can confirm that these polymer epitopes co-elute in our system. Currently further confirmation is needed such as using NMR spectroscopy to prove covalent linkages exist in a purified co-polymer. Moreover, this AGP co-polymer could have important implications for wine colour, enzyme processing and sensory impacts that deserve future research investigations.

This study was limited by the number of samples that could have been realistically analysed, as well as the difficulty of obtaining commercial wine samples from the same year. Additionally other analytical techniques are still needed to focus on smaller polymers such as RGI and

RGII. A larger sample size that is more focused on either harvest effect, vineyard conditions, or other factors could clarify these effects of polysaccharide size and epitope distribution. Further investigation of the AGP-RG-I-XyG co-polymer could focus on the cleavage of the RG-I backbone to attempt to completely break the polymer apart, yielding clues to more detailed structural information.

CRediT authorship contribution statement

Brock Kuhlman: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing; **Jose Luis Aleixandre-Tudo:** Conceptualization; Supervision; Writing - review & editing; **Wessel du Toit:** Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing - review & editing; **John P. Moore:** Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing - review & editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2023.109100.

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