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Quantification of major grape polysaccharides (*Tempranillo* v.) released by maceration enzymes during the fermentation process

B. Ayestarán*, Z. Guadalupe, D. León

Departamento de Agricultura y Alimentación, Universidad de La Rioja, Madre de Dios 51, E-26006 Logroño, La Rioja, Spain

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Abstract

The influence of commercial enzymes on wine polysaccharide content was studied. Tempranillo wines were made using commercial maceration enzyme preparations along with controls. The analytical method for the quantification of wine polysaccharides was carried out by a multistep procedure. Wine-soluble polysaccharides were isolated by wine concentration polysaccharides precipitation with an acid–alcohol medium and separation of each polysaccharide family by high resolution size-exclusion chromatography on a Superdex-75 HR column. The glycosyl-residue compositions of the fractions obtained were determined by gas chromatography with flame ionisation and mass spectrometry of their trimethylsilyl-ester *O*-methyl glycosides after acidic methanolysis and derivatization. The content of each fraction was estimated from the concentration of individual glycosyl residues that are characteristic of well-defined wine polysaccharides. The analytical method proposed had good sensitivity, repeatability, reproducibility and accuracy. Soluble polysaccharides in wine were essentially composed of grape cell wall polysaccharides: arabinogalactans and arabinogalactan-proteins (38–41%), and rhamnogalacturonans-II (38–46%). Yeast mannans and mannoproteins were also present but in smaller proportions (14–19%). Wines treated with commercial enzymes had larger concentrations of arabinogalactans, arabinogalactan-proteins and rhamnogalacturonans-II than control wines, but the content of mannans and mannoproteins was similar in both wines. This indicated that the commercial enzymes hydrolysed grape pectic polysaccharides during the maceration–fermentation stage but had no influence on yeast parietal polysaccharides.

Keywords: Wine; Polysaccharides; Maceration enzymes; Polysaccharide analysis

1. Introduction

Polysaccharides are one of the main groups of wine macromolecules. They are considered as "protective colloids", liable to prevent or limit aggregation, flocculation and thereby haze formation and tartrate salts crystallisation [1,2]. Wine polysaccharides have also been described for their detrimental role in filterability [3–5], their influence on the fermentation flora [6–8] and their interaction with aromatic compounds [9,10]. These compounds also contribute to the organoleptic properties of wines, as they stabilise flavour, colour and foam [11].

Wine polysaccharides originate from both grape primary cell walls (pectic polysaccharides) and yeast cell walls (mannoproteins and mannans) [12]. Hence, this origin diversity leads to polysaccharide families that are different in composition and structure. Two criteria widely used for polysaccharide families discrimination are acidity and protein content [10]. Neutral pectic substances mainly comprise type II arabinogalactans (AGs) and arabinogalactan-proteins (AGPs), which represent more than 40% of total red wine polysaccharides [13,14]. Their common structural feature is a (1 → 3)-β-D-galactan backbone with (1 → 6) linked β-D-galactan side chains highly substituted by arabinofuranosyl residues. Typical AGPs commonly contain less than 10% protein [14]. Other neutral polysaccharides are weakly branched (1 → 5)-α-L-arabinans [15] and type I arabinogalactans that are (1 → 4)-β-D-galactans substituted in position 6 by arabinofuranosyl residues. Acidic pectic polysaccharides, characterised by a high proportion of galacturonic acid, involve homogalacturonans and rhamnogalacturonans. Rhamnogalacturonans-II (RG-IIs), which represent about 20% of soluble polysaccharides in red wine, are (1 → 4)-α-D-galacturonans branched with four

* Corresponding author. Fax: +34-941-299721.
E-mail address: belen.ayestaran@daa.unirioja.es (B. Ayestarán).

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different side chains containing primarily rhamnopyranose, arabinofuranose and galactopyranose [16,17].

Mannans and mannoproteins are produced by yeast, such as *S. cerevisiae*, during alcoholic fermentation [13,18]. Pectic polysaccharides arise from native cell wall pectines of grape berry after degradation by pectinases during grape maturation and during the first steps of their processing to wine [19]. Commercial enzymes have been traditionally used in wine technology in order to produce even higher modifications in grape polysaccharides than those produced by endogenous pectinases. Ducruet et al. [20] observed that the addition of commercial enzymes to musts produced an increase in the amount of total acid (49%) and neutral polysaccharides (5%), but they did not study this effect in the different polysaccharide families.

The identification and structural characterisation of each polysaccharide family require its previous isolation from total wine macromolecules, followed by ion-exchange, size-exclusion and affinity chromatographies [3,16,21,22]. The methods most commonly used for extraction are precipitation with ethanol, dialysis and ultrafiltration [23]. Several methods for the direct quantification of polysaccharides in wines have been proposed. Most of these are based on the precipitation of total wine colloids, followed by colorimetric assays [3,18,21,23–26] or by the determination of peak areas in size-exclusion chromatography [27–29]. However, these global methods do not allow the identification of the different families of polysaccharides present in wines. Because of their diversity and chemical complexity, the identification and quantification of wine polysaccharide families requires a series of complex analytical procedures. However, reliable quantification may be achieved, from the concentration of individual monosaccharides that are characteristic of well-defined wine polysaccharides. Many chromatographic methods have been proposed for the identification and quantification of carbohydrates [30]. Due to their high sensitivity combined with an ability to achieve efficient separation of complex mixtures, gas chromatography (GC) and GC-mass spectrometry (GC-MS) have gained general acceptance [31]. The neutral glycosyl-residue compositions of plant polysaccharides are typically determined, after acid hydrolysis, by GC and GC-MS analysis of their aditol acetate derivatives [32] but the acidic glycosyl-residue composition cannot be determined directly by this procedure.

In the present study, published references about all these methodologies were revised. The most appropriate steps of the revised methods, to the best of our knowledge, were studied in detail. The analytical method for the quantification of wine polysaccharides was carried out in a multistep procedure. This method was applied to quantify the major family grape polysaccharides released by commercial enzymes during the maceration–fermentation of the Tempranillo grape. The properties of the proposed method were studied in detail and a validation study was carried out.

2. Experimental

2.1. Reagents and samples

All reagents were of analytical grade unless otherwise stated. L-Fucose, L-rhamnose, 2-*O*-methyl-D-xylose, L-arabinose, D-xylose, D-galactose, D-glucose, D-mannose and Kdo (3-deoxy octulosonic acid) were supplied by Sigma (St. Louis, MO), and D-galacturonic acid, D-glucuronic acid and myo-inositol from Fluka via (Sigma). D-apiose was obtained from Omicrom (South Bend, IN).

Ethanol (96%, v/v) and acetyl chloride were supplied by Scharlab (Barcelona, Spain), hydrochloric acid 37% by Carlo Erba (Rodano, Milan, Italy), dried methanol Merck (Darmstadt, Germany) and trimethylsilylation reagent (TriSil®) by Pierce (Rockford, MA). HPLC-grade ammonium formiate supplied by Fluka and Milli-Q water (Millipore, Molsheim, France) were used. A pullulan calibration kit (Shodex P-82) was obtained from Waters (Barcelona, Spain). All the solutions were filtered through a 0.45 μm filter before use in liquid chromatography (LC).

Wine samples were produced from *Vitis Vinífera Tempranillo* grapes of the qualified origin denomination Rioja (D.O.Ca Rioja). The yeast *S. cerevisiae* RC 212 and the maceration enzymes were purchased from Lalvin (Lallemand Inc., Montreal, Canada).

2.2. Vinification and sample collection

Six experimental vinifications were carried out using stainless steel tanks of 100 l. Destemmed-crushed grapes were homogenised and distributed into the tanks, 30 mg l^{-1} SO_2 was added and yeast *S. cerevisiae* RC 212 inoculated. After 1 h, 0.02 g l^{-1} maceration enzymes were added to three of the tanks and the musts were mixed thoroughly. These enzymes showed standard activities >4000 uPG g^{-1} (polygalacturonase units), 1000 uPE g^{-1} (pectin-esterase units) and 120 uPL g^{-1} (pectin-liase units). Vinifications without enzymes were considered as control treatments.

In the prefermentation stage, the initial measurements of pH, g tartaric acid/100 ml and g l^{-1} reducing sugars, were 3.5, 0.6 and 230, respectively. The fermentation–maceration process was carried out at a maximum temperature of 28 ± 2 °C and went on for 10 days. *S. cerevisiae* RC 212 was implanted in all the vinifications and this was corroborated by molecular biological techniques (PCR and EPC).

Wine samples were taken at the end of maceration–fermentation, when 99% of sugars had been consumed, and were collected from both enzyme-treated tanks (EXV wine) and control tanks (control wine). Sample bottles were filled completely to minimise oxygen contact and immediately frozen at −18 °C. All samples were analysed for titratable activity, pH, percent of alcohol, total phenols and reducing sugars prior to freezing.

2.3. Preparation of wine polysaccharides

Samples were homogenised and 400 ml was taken with a peristaltic pump and centrifuged (9500 × *g*, 20 min, 4 °C) using a RC-5B Sorvall refrigerated centrifuge (Du Pont, BH, Germany).

The insoluble pellets were recovered and precipitated with 50 ml of cold 96% ethanol containing 0.3 M HCl. After 18 h at 24 °C, samples were centrifuged (9000 × *g*, 20 min, 4 °C) and the pellets obtained washed in ethanol 96% several times (until the supernatant was colourless) to remove interfering materials. The residues obtained (fraction A) were freeze-dried using a Virtis freeze drying (New York, NY).

The supernatants were first concentrated five times under reduced pressure at 34 °C. Total soluble polysaccharides were then precipitated by adding of four volumes of cold ethanol containing 0.3 M HCl, and kept for 18 h at 4 °C. Thereafter, the samples were centrifuged (9000 × *g*, 20 min, 4 °C), the supernatants discarded and the pellets washed four times with ethanol 96%. The precipitates were finally dissolved in ultrapure water and freeze-dried (fraction B).

2.4. Fractionation of soluble polysaccharides by high resolution size-exclusion chromatography

In order to separate the different polysaccharide families, the soluble fractions B were submitted to high resolution size-exclusion chromatography (HRSEC). Four mg of freeze-dried fraction B were dissolved in 1 ml of ultrapure water and centrifuged (4000 × *g*, 5 min, 4 °C) to remove the insoluble material prior to analysis.

HRSEC was performed using an Agilent modular 1100 liquid chromatograph (Waldbronn, Germany) equipped with one G1310A HPLC pump, an on-line G1379A degasser, a G1362 refractive index detector, and a Windows 2000 Hewlett-Packard computer, and furnished with a Superdex-75 HR column (1.3 cm × 30 cm, Pharmacia, Sweden). Samples were injected using a manual injector (Rheodyne, CA) and collected in a Gilson fraction collector (Middletown, WI).

The mobile phase used was 30 mM ammonium formiate, pH 5.8; the flow rate and the injection volume were 0.6 ml min^{-1} and 500 μl, respectively. Chromatographic separation was carried out at room temperature.

The peaks obtained were collected in different fractions according to their elution times: fraction B1 (12–17 min), fraction B2 (18–22 min) and fraction B3 (25–30 min). The isolated fractions were freeze-dried, redissolved in water, and freeze-dried again four times to remove the ammonium salt. Each sample was injected at least 20 times in order to obtain enough freeze-dried quantities for further analysis.

The molecular weight distribution of the different fractions was determined by calibration of the Superdex-75 HR column with a pullulan calibration kit. Chromatographic separation of the pullulan standards was performed under the same conditions described above.

2.5. Identification and quantification of polysaccharides by GC and GC-MS

The carbohydrate composition of the fractions (fractions A, B and B1–B3) was determined by GC with flame ionisation detector and GC-MS of their trimethylsilyl-ester *O*-methyl glycolsyl-residues (TMS) obtained after acidic methanolysis and derivatization of these fractions. This derivatization procedure allows the identification of both neutral and acidic monosaccharides.

The polysaccharide contents of fractions B1, B2 and B3 were estimated from the concentration of individual glycosyl residues that are characteristic of well-defined wine polysaccharides.

2.5.1. Sample preparation: acidic methanolysis and derivatization

Polysaccharide fractions were treated with the methanolysis reagent MeOH 0.5 M HCl in order to hydrolyse neutral and acidic monosaccharides to their corresponding methyl glycosides. The methanolysis reagent was prepared by adding acetyl chloride (140 μl) to 4 ml of dried methanol. Freeze-dried samples (0.5–1 mg) and 1 mg of inositol (internal standard) were hydrolysed with 0.5 ml of the methanolysis reagent. The reaction was conducted under reduced pressure at 80 °C for 16 h. Thereafter, the excess of reagent was removed using a stream of nitrogen gas.

The conversion of the methyl glycosides to their trimethylsilyl (TMS) derivatives was performed using the TriSil® reagent. An excess of TriSil® reagent (0.3 ml) was added to the dried material. The reaction was carried out at 80 °C for 20 min and the reagents again removed with a nitrogen stream. The derivatized residues were then extracted with 1 ml of hexane, evaporated to dryness with a nitrogen stream, and mixed again with 40 μl of hexane. GC-FID and GC-MS were performed with 2 μl of these solutions. All analyses were performed in triplicate.

Different quantities of standard carbohydrates (0.1–5 mg) were also converted to their corresponding TMS derivatives and analysed by GC and GC-MS in order to obtain patterns for identification and standard calibration graphs. The procedure followed with the standards was the same as with the fraction samples but the derivatized residues were extracted with 2 ml of hexane, and 1 μl was used for GC and GC-MS analysis.

2.5.2. Gas chromatography

The GC system consisted of an HP5890 Series II gas chromatograph (Hewlett-Packard, USA) coupled to a FID. The GC system was equipped with a capillary split/splitless inlet and a fused-silica capillary column (30 m × 0.25 mm × 0.25 μm, Teknokroma). The carrier gas was helium at a flow rate of 1.3 ml min^{-1}. Samples were injected in the pulsed split mode with a split ratio of 20:1. The injector and the FID were operated at 250 °C. The chromatograph was operated with temperature programming (120–145 °C

at 1 °C min^{-1}, 145–180 °C at 0.9 °C min^{-1} and 180–230 °C at 50 °C min^{-1}).

2.5.3. Gas chromatography-mass spectrometry

GC-MS was performed with a Hewlett-Packard HP-G1800B GCD coupled to a mass detector operated in the electron ionisation (EI) mode under the control of a GCD Plus Chemstation Agilent, G 2070. Chromatographic separation was performed under the same conditions described above but the flow rate was 1 ml min^{-1}. EI mass spectra were obtained over the range *m/z* 50–450 every 2.8 s in the total ion-monitoring mode using a source of temperature 230 °C, a quadrupole temperature of 136 °C, and a ionisation voltage of 70 eV.

3. Results and discussion

3.1. Preparation of wine polysaccharides

Soluble polysaccharides were isolated from total wine macromolecules by precipitation with an acid–alcohol medium. In order to select the best conditions for this precipitation from those proposed by other authors [3,6,9,21,23–26,33–35], several experiments were done in order to optimise the precipitation time and temperature.

Aliquots (5 ml) of centrifuged wine sample were precipitated with four volumes of ethanol–acid for different times (6, 10, 14, 18, 22 and 26 h) and at different temperatures (4 °C and ambient temperature). The precipitates were washed with ethanol, as described in the above method, freeze-dried and weighed. Table 1 shows the means and standard deviations of the quantities obtained. As can be observed in this table, the quantity of polysaccharide precipitate increased with the time of precipitation up to 18 h and after this time the weight was constant. Larger amounts of precipitate were obtained at 4 °C in comparison with ambient temperature. Thus, 4 °C and 18 h were chosen as precipitation conditions.

In order to isolate the polysaccharides present in the insoluble pellets (fraction A), these fractions were also precipitated with ethanol–acid. Although this precipitation is not normally done by other authors when treating with insoluble fractions [35], non-precipitated pellets contain a very large quantity of insoluble wine macromolecules, such proteins, and tannins, which could cause interferences when measuring carbohydrate content. It was observed that the weight of non-precipitated pellets was almost double than that of the precipitated ones, but the amount of total polysaccharides, measured by the colorimetric methods of phenol and *o*-hydroxydiphenyl [23], was higher in the latter.

The supenatants (fraction B) obtained after removal of insoluble pellets were concentrated prior to the addition of cold ethanol–acid. This was necessary to ensure the quantitative precipitation of all soluble polysaccharides since some polysaccharide families (rhamnogalacturonans and homogalacturonans) are precipitated only partially in non-concentrated wines [35]. Several authors have analysed polysaccharide families in concentrated wine samples [3,14,35,36] and the concentrations used differed from one author to another. Similar studies made in musts focus on non-concentrated samples [12,37]. As there were different criteria for the concentration rate, and since no previous studies of the influence of sample concentration on polysaccharide precipitation were found it was considered interesting to analyse this in detail. Must and wine samples were centrifuged, the supernatants concentrated different times, precipitated, and submitted to HRSEC analysis. The chromatograms obtained are shown in Figs. 1 and 2.

Must samples were concentrated zero, two and three times. It was impossible to concentrate them more than three times because the polysaccharides began to caramelise in the media and became non-miscible with the precipitation reagent. Wine samples were also concentrated different times (0-, 2-, 4-, 5-, 7- and 10-fold).

In both musts and wines, the quantity of polysaccharide precipitate obtained after the precipitation procedure increased with the sample concentration rate (Table 2), indicating that part of the polysaccharides did not precipitate in less concentrated samples, probably due to their high solubility.

Non-concentrated musts showed only one peak in the HRSE chromatogram whereas two-fold concentrated samples also showed a second peak, and three peaks were obtained in must samples concentrated three times (Fig. 1). Thus, it could be concluded that it was necessary to concentrate must samples three times in order to obtain the three peaks and avoid loosing some polysaccharide families, as RG-IIs and homogalacturonans mainly elute in these fractions [35]. In wines, non-concentrated and two-fold

Table 1
Polysaccharide precipitate concentration[a] (mg l^{-1}) found in non-concentrated wine samples precipitated for various times at two temperatures

Precipitation temperature	Precipitation time (h)					
	6	10	14	18	22	26
Ambient	448 ± 32	490 ± 46	658 ± 41	756 ± 25	728 ± 62	745 ± 51
4 °C	470 ± 28	554 ± 56	742 ± 48	848 ± 54	890 ± 62	863 ± 62

[a] Mean ± S.D. ($n = 6$).

Table 2
Polysaccharide precipitate concentration[a] (mg l^{-1}) found in wine and must samples concentrated a different number of times

Sample	Times concentrated							
	0	2	3	4	5	6	7	10
Must	215 ± 18	368 ± 42	874 ± 61	–	–	–	–	–
Wine	423 ± 35	435 ± 56	–	761 ± 58	896 ± 62	–	991 ± 71	1278 ± 123

Precipitation carried out at 4 °C for 18 h.
[a] Mean ± S.D. ($n = 6$).

concentrated samples showed only one and two peaks, respectively, whereas the rest of the concentrated samples showed the three fractions needed for the quantification of all polysaccharide families (Fig. 2). These three peaks showed higher resolution in the samples concentrated five times and, therefore, and also in order to minimise the concentration time, this concentration was chosen for wine samples.

The repeatability of the polysaccharide precipitation method was also analysed in both wines and musts. Eight wine samples and eight must samples were centrifuged and the supernatants concentrated and precipitated. The residues were washed with ethanol, freeze-dried and weighed. Repeatability was expressed as the coefficient of variation of the precipitate quantities obtained and was 3.02% for wine samples and 2.89% for must samples. These values showed the precision of the method under the conditions selected.

3.2. Fractionation of soluble polysaccharides by HRSEC

HRSEC was carried out using a Superdex-75 HR column. This pre-packed column, with a molecular weight range from 3000 to 75,000 Da, was used for the fractionation of wine-soluble polysaccharides. In the wine samples

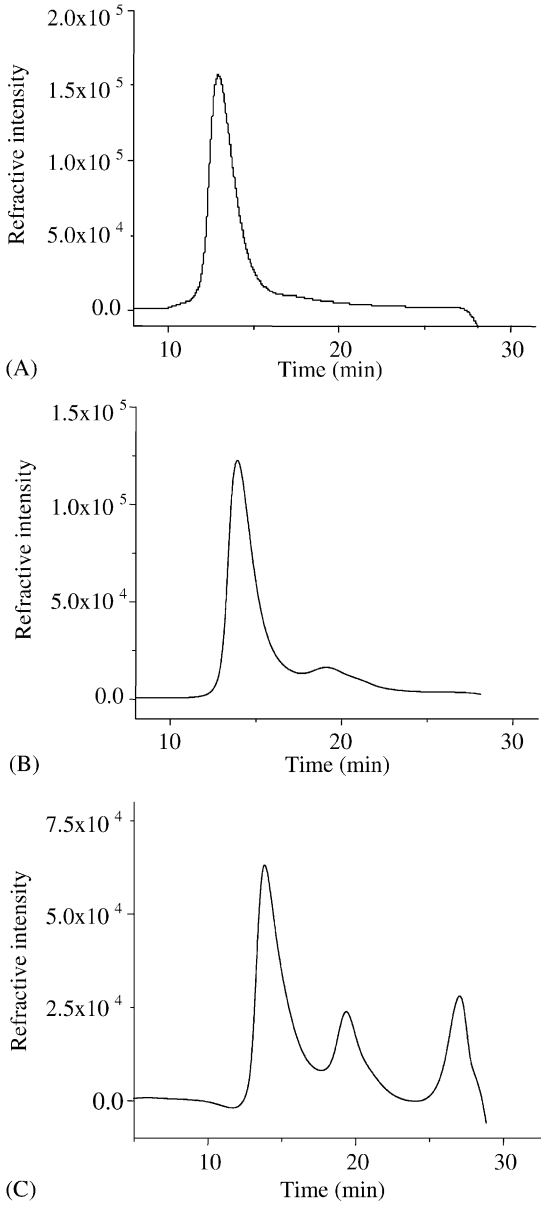

Fig. 1. HRSEC profiles of soluble polysaccharides in must samples concentrated a different number of times. (A) Non-concentrated must sample. (B) Must sample concentrated two-fold. (C) Must sample concentrated three-fold.

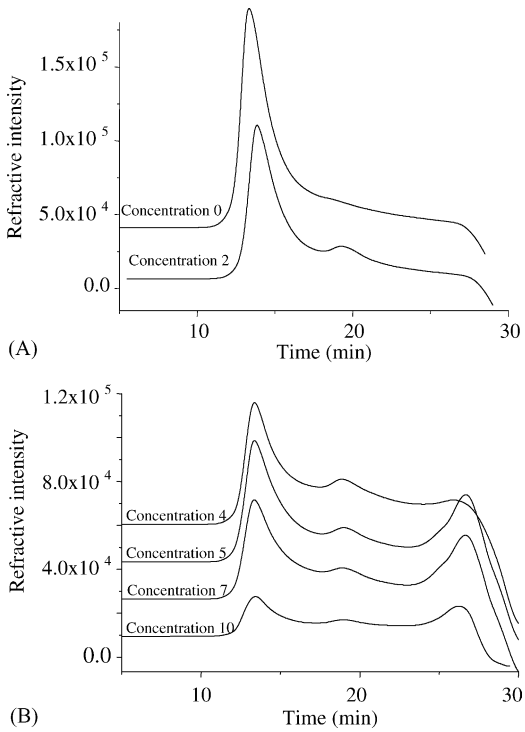

Fig. 2. HRSEC profiles of soluble polysaccharides in wine samples concentrated a different number of times. (A) Wine samples concentrated zero- and two-fold. (B) Wine samples concentrated 4-, 5-, 7- and 10-fold.

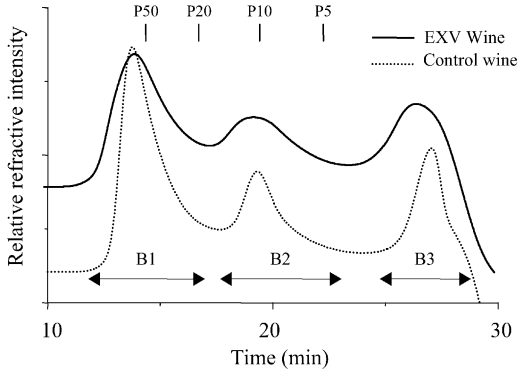

Fig. 3. Molecular weight distribution of fractions B1, B2 and B3 obtained by HRSEC on a Superdex-75 HR column. Elution times of pullulan standards (P5 → P50) are also shown.

analysed, the HRSEC fractionation allowed the separation of soluble polysaccharides into three different fractions: B1, B2 and B3. The molecular weight distribution of these fractions and the refractometric profiles are shown in Fig. 3. The population eluting between 11 and 17 min (fraction B1) corresponded to molecules with a molecular weight > P20 (22,800 Da). According to previously published data, these molecules correspond to arabinogalactans, arabinogalactan-proteins, mannans and mannoproteins [19,35]. A second population (fraction B2), with an average molecular weight between P20 and P5 (22,800 and 6000 Da), eluted between 17 and 24 min. This population correspond to a complex mixture of mainly RG-II dimers (average molecular weight ∼10,000 Da) [19,35]. The third population (fraction B3), with a weight < P5 (6000 Da), eluted between 26 and 32 min and could be attributed to oligosaccharides and low molecular weight fragments of very large macromolecules.

The reproducibility and repeatability of the HRSEC fractionation (Table 3) was assessed by analysing the results obtained with each sample; 20 injections per sample were carried out. The peak area repeatability, expressed as the coefficient of variation of each sample, was <5% in all samples. The coefficient of variation of the retention time was <0.6% in all cases. The peak area reproducibility, expressed as the mean of the coefficients of variation of all the measures, was 3 ± 1%. This value was 0.2 ± 0.1% for the retention time.

Table 3
Reproducibility of relative concentrations and retention times of the fractions obtained by HRSEC on a Superdex-75 HR column

Sample	Fraction	Relative concentration[a] (%)	Retention time[b] (min)
Control wine	B1	50.1 ± 0.6	13.85 ± 0.07
	B2	27.5 ± 0.9	19.40 ± 0.03
	B3	22.4 ± 0.8	27.09 ± 0.06
EXV wine	B1	43.7 ± 0.6	14.02 ± 0.04
	B2	29.5 ± 0.6	19.43 ± 0.02
	B3	26.9 ± 0.5	26.98 ± 0.05

[a] Calculated on the basis of total recovered carbohydrates. Mean±S.D. ($n = 20$).
[b] Mean ± S.D. ($n = 20$).

3.3. Identification and quantification of glycosyl residues by GC and GC-MS

The monosaccharide composition of all the fractions (A, B, B1, B2 and B3) was first determined by GC and GC-MS of their TMS residues. In order to be able to identify and quantify the monosaccharides in the gas chromatograms (Fig. 4), calibration graphs of sugar standards were required. L-Fucose, L-rhamnose, 2-*O*-methyl-D-xylose, L-arabinose, D-xylose, D-galactose, D-glucose, D-mannose, Kdo, D-galacturonic acid, D-glucuronic acid and D-apiose were used as monosaccharide standards, myo-inositol was used as internal standard. The equation, slope and intercept standard deviations, the correlation coefficients (r) and the limits of detection (LD) and quantification (LQ), for the carbohydrate standards are shown in Table 4. In addition, a recovery study of each standard was carried out (Table 4). The correlation coefficients obtained from the linear calibration graphs were all ≥ 0.992 ($P < 0.001$). These curve were therefore, were considered to be linear for the range of amounts studied (0–5 mg). The LDs and LQs showed an acceptable sensitivity; all the values obtained for the monosaccharides present in the fractions were above these limits. The recovery results showed the precision of the calibration curves.

GC-MS was used to identify those monosaccharides for which no commercial standards were available: 2-*O*-methyl fucose, aceric acid and Dha (3-deoxy-D-*lyxo*-heptulosaric acid). The identification of the peaks in the chromatogram was based on their GC retention times and MS fragmentation patterns reported in [38]. These carbohydrates were quantified using the 2-*O*-methyl xylose calibration curve.

3.4. Validation of the proposed method

The applicability of the method was checked by analysing real wine samples. Repeatability was evaluated by analysing of 10 wine aliquots under normal operating conditions. Wine samples were centrifuged and the supernatants concentrated and precipitated. The residues obtained were freeze-dried, methylated, derivatizated and submitted to GC and GC-MS analysis. The amount of D-galactose, D-glucose and D-mannose, major wine carbohydrates, was quantified in each aliquot and the repeatability expressed as the coefficient of variation ($n = 10$). The values obtained were 2.4% for galactose, 3.8% for glucose and 3.2% for mannose. The reproducibility of the method was calculated as the mean of the coefficients of variation of each of the samples analysed, from three replicate measurements. The same sugars described above were determined and the values obtained were 2.0 ± 0.6% for galactose, 4.2 ± 1.2% for glucose and 3.8 ± 0.9% for mannose. In addition, a recovery study of galactose was carried out in order to assess the accuracy

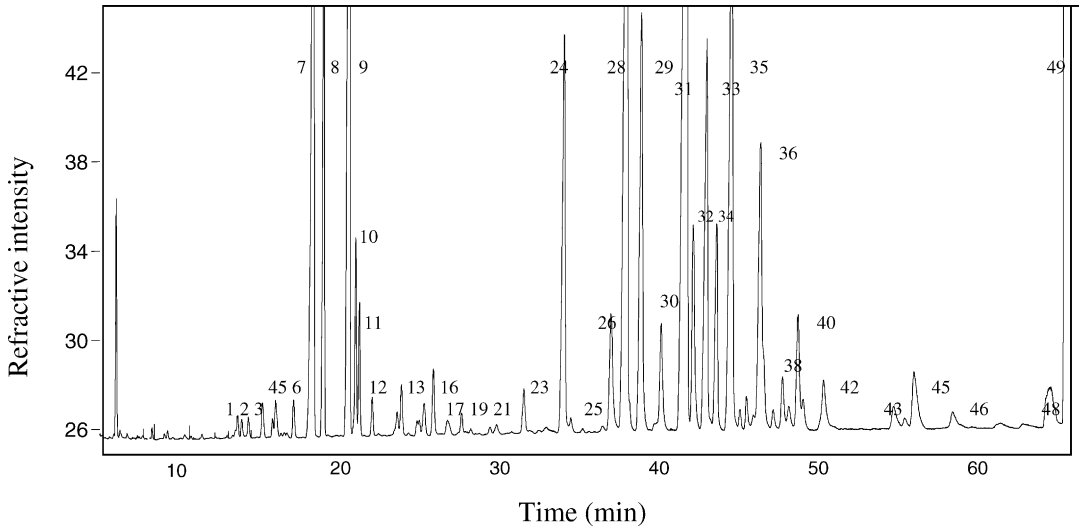

Fig. 4. GC-FID profiles of the glycosyl residues of red wine polysaccharides (fraction B1 of the control wine). Aceric acid (peaks 1, 2), 2-*O*-Me-fucose (peak 3), 2-*O*-Me-xylose (peaks 4, 5), apiose (peaks 6–8), arabinose (peaks 7, 8, 11, 15), rhamnose (peaks 9–11), fucose (peaks 10, 12, 13), xylose (peaks 14, 16, 21, 25), galacturonic acid (peaks 17, 24, 26, 33, 34, 39, 41), glucuronic acid (peaks 22, 37, 38, 42), Dha (peaks 27, 30, 32), mannose (peaks 28, 30), galactose (peaks 29, 31, 32, 35), Kdo (peaks 34, 45, 47, 48), glucose (peaks 36, 40, 44, 46), inositol (peak 49).

of the method. Ten wine samples were analysed to give the amount of galactose present and analysed again after the addition of two different quantities (1 and 2 mg) of this monosaccharide. The recovery obtained was 99 ± 4%.

3.5. Analysis of insoluble fraction A and soluble fraction B

The carbohydrate compositions of fractions A and B are shown in Table 5.

The carbohydrate content of insoluble fractions A was quite similar in both wines analysed. Glucose, a constituent of condensed polyphenolic compounds and microbial cell walls [35,39], was the main sugar detected in fractions A, representing >42% of total insoluble polysaccharides. Mannose, the main component of yeast mannans and mannoproteins [40–42], was also found in large amounts. The other sugars detected were galactose, arabinose and rhamnose, the glycosyl residues found in arabinogalactans [14]. Galacturonic acid, the main component of homogalacturonans and rhamnogalacturonans [10], was also present in small amounts. The insoluble polysaccharides present in fractions A represented quite an important amount of total wine polysaccharides, 44% for the control wine and 29% for the wine treated with enzymes (EXV wine). Thus, the precipitation of polysaccharides is an important phenomenon during the fermentation–maceration stage. This polysaccharide insolubilisation, due to the effect of ethanol, affects mainly mannans, mannoproteins, arabinogalactans and arabinogalactan-proteins.

Table 4
Monosaccharide standard calibration parameters and validation results for GC-FID

Sugar	Equation[a]	S.D.$_{slope}$ ($n = 10$)	S.D.$_{intercept}$ ($n = 10$)	LD (μg)	LQ (μg)	r^2[b]	Recovery (%) ($n = 5$)
Fucose	$A = 0.496C - 0.004$	0.016	0.010	<1	5	0.997	103 ± 4
Rhamnose	$A = 0.642C - 0.009$	0.032	0.012	<1	<1	0.994	103 ± 7
2-*O*-Me Xyl[c]	$A = 0.294C - 0.002$	0.006	0.003	<1	7	0.998	101 ± 2
Arabinose	$A = 0.449C - 0.006$	0.035	0.004	<1	4	0.992	98 ± 5
Galactose	$A = 0.401C - 0.022$	0.010	0.025	<1	<1	0.999	101 ± 4
Xylose	$A = 0.4616C - 0.0003$	0.017	0.001	3	10		99 ± 5
Glucose	$A = 0.857C - 0.089$	0.007	0.020	14	21	0.999	99 ± 2
Mannose	$A = 0.828C - 0.009$	0.071	0.002	<1	1	0.999	100 ± 2
Kdo[c]	$A = 0.0812C - 0.0009$	0.004	0.002	2	9	0.997	97 ± 5
GalA[c]	$A = 0.250C - 0.003$	0.001	0.004	<1	<1	0.999	100 ± 2
GlcA[c]	$A = 0.282C - 0.003$	0.017	0.010	<1	7	0.999	100 ± 1
Apiose	$A = 0.224C - 0.005$	0.011	0.007	2	9	0.992	97 ± 5

[a] A and C denote the peak area and amount in mg, respectively.
[b] Linear correlation coefficient for 0–5 mg ($n = 8$).
[c] 2-*O*-Me Xyl, 2-*O*-methyl xylose; Kdo, 3-deoxy octulosonic acid; GalA, galacturonic acid; GlcA, glucuronic acid.

Table 5
Carbohydrate composition (mg l^{-1}) of fractions A and B determined by GC and GC-MS of their TMS derivatives

Sugars	Control wine		EXV wine	
	Fraction A	Fraction B	Fraction A	Fraction B
2-*O*-Me Fuc[a]	–[b]	16 ± 1	–	37 ± 1
Rhamnose	39.9 ± 0.2	50.7 ± 0.3	40.0 ± 0.1	87.5 ± 0.3
Fucose	–	16.5 ± 0.3	–	39.2 ± 0.4
2-*O*-Me Xyl[a]	–	16 ± 1	–	38 ± 1
Arabinose	42.5 ± 0.3	134 ± 1	47 ± 1	168 ± 1
Xylose	–	3.9 ± 0.2	–	7.8 ± 0.2
Apiose	–	38.8 ± 0.2	–	8.9 ± 0.2
Mannose	151 ± 1	134 ± 1	119 ± 1	194 ± 2
Galactose	152 ± 1	193 ± 1	157 ± 1	369 ± 1
Glucose	358 ± 2	119 ± 1	304 ± 2	270 ± 1
GalA[a]	52.6 ± 0.1	64 ± 1	48 ± 1	117 ± 2
GlcA[a]	–	23.1 ± 0.1	–	54 ± 1
Kdo[a]	–	110 ± 1	–	231 ± 2
Dha[a]	–	59.6 ± 0.4	–	125 ± 1
AceA[a]	–	38.8 ± 0.2	–	8.9 ± 0.2
Total	796 ± 2	1017 ± 3	715 ± 3	1755 ± 4

[a] 2-*O*-Me Fuc, 2-*O*-methyl fucose; 2-*O*-Me Xyl, 2-*O*-methyl xylose; GalA, galacturonic acid; GlcA, glucuronic acid; Kdo, 3-deoxy octulosonic acid; Dha, 3-deoxy-D-*lyxo*-heptulosaric acid; AceA, aceric acid.
[b] <1 mg l^{-1}.

The soluble fractions B contained all the sugars that form wine polysaccharides. Large quantities of mannose, arabinose, galactose, rhamnose, glucuronic and galacturonic acid were found and several rare sugars, such as apiose, fucose, 2-*O*-methyl L-fucose, 2-*O*-methyl D-xylose, aceric acid (3-*c*-carboxy-5-deoxy-L-xylose), Kdo (3-deoxy octulosonic acid), and Dha (3-deoxy-D-*lyxo*-heptusolaric acid) were also quantified. These rare sugars are known as markers of the RG-II molecule [16]. The presence of all these glycosyl residues confirmed the predominance of mannans, mannoproteins, arabinogalactans, rhamnogalacturonans and RG-II molecules in red wines [43]. The presence of glucose in fractions B could be attributed to microbial polysaccharides and condensed anthocyanins [35]. The presence of xylosyl residues indicated that traces of hemicelluloses (arabinoxylans or xyloglucans) might also be solubilised from grape cell walls [35].

The quantity of total soluble monosaccharides of fractions B was higher in the wine treated with enzymes (EXV wine) than in the control one, indicating that the commercial enzymes had greater effects on wine carbohydrates than the endogenous ones.

3.6. Estimation of polysaccharide concentrations

Wine fractions B were submitted to HRSEC, and three different fractions were obtained: B1, B2 and B3. The monosaccharide composition of these fractions (Table 6) was determined by GC and GC-MS of their TMS residues as described previously. The polysaccharide content of each fraction (Table 7) was estimated from the concentration of individual glycosyl residues that are characteristic of well-defined wine polysaccharides.

High molecular weight polysaccharides, collected in fraction B1, were composed mainly of mannose, arabinose, galactose, rhamnose and glucuronic acid (Table 6), confirming the predominance of mannans and mannoproteins (M) and arabinogalactans and arabinogalactan-proteins (AGs) among wine polysaccharides. Arabinogalactans (AGs) are mainly composed of galactose and arabinose and minor amounts of rhamnose and glucuronic acid [14]. The molecular ratios of these residues depend on type and the hydrolysis rate of the molecule [14,22,44]. AGs were therefore estimated from the sum of galactosyl, arabinosyl, rhamnosyl and glucuronosyl residues. All the mannose content was attributed to yeast mannans and mannoproteins (M).

The composition of fraction B2 was more complex and all the rare diagnostic sugars of the RG-II molecule were detected (Table 6), confirming the predominance of this polysaccharide. These characteristic sugars included apiose, 2-*O*-methyl-L-fucose, 2-*O*-methyl-D-xylose, aceric acid (3-*c*-carboxy-5-deoxy-L-xylose), Kdo (3-deoxy octulosonic acid), and Dha (3-deoxy-D-*lyxo*-heptusolaric acid). However, the molar ratios of arabinosyl, rhamnosyl and galactosyl residues were greater than expected for a purified RG-II molecule [17,45], and mannose and glucuronic acid were also present in these fractions (Table 6), indicating the presence of low molecular weight arabinogalactans, mannans and mannoproteins. The RG-II content was calculated from the sum of its diagnostic sugars, which represent approximately 25% of the RG-II molecule [35]. For one residue of 2-*O*-methyl fucose, RG-II contains 5 rhamnosyl, 3 arabinosyl, 2 galactosyl and 10 galacturonosyl residues [17,45]. Taking into account these molar ratios, it was possible to estimate their respective amounts in the RG-II. The remaining part was attributed to the

Table 6
Carbohydrate composition (mg l^{-1}) of fractions B1, B2 and B3 obtained by HRSEC on a Superdex-75 HR column and determined by GC and GC-MS of their TMS derivatives

	Control wine fractions			EXV wine fractions		
	B1	B2	B3	B1	B2	B3
2-*O*-Me Fuc[a]	–[b]	6.0 ± 0.1	4.6 ± 0.1	–	10.6 ± 0.1	4.9 ± 0.1
Rhamnose	18.9 ± 0.4	20.8 ± 0.2	8.9 ± 0.1	17.2 ± 0.3	32.6 ± 0.2	11.1 ± 0.1
Fucose	6.0 ± 0.1	5.7 ± 0.1	4.4 ± 0.1	6.9 ± 0.2	9.9 ± 0.1	4.8 ± 0.1
2-*O*-Me Xyl[a]	–	6.2 ± 0.1	4.8 ± 0.1	–	11.3 ± 0.1	4.8 ± 0.1
Arabinose	37.4 ± 0.8	40.0 ± 0.4	13.5 ± 0.2	124 ± 1	65.9 ± 0.5	17.1 ± 0.1
Xylose	2.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	2.2 ± 0.1	1.9 ± 0.1	1.5 ± 0.1
Apiose	–	14.2 ± 0.1	9.5 ± 0.1	–	24.4 ± 0.2	10.0 ± 0.1
Mannose	106.5 ± 2.2	18.1 ± 0.2	16.9 ± 0.2	114 ± 3	36.2 ± 0.3	21.4 ± 0.3
Galactose	141.6 ± 2.9	50.9 ± 0.5	34.9 ± 0.4	142 ± 3	89.6 ± 0.6	42.6 ± 0.3
Glucose	4.6 ± 0.8	30.1 ± 0.3	41.6 ± 0.5	5 ± 1	49.0 ± 0.4	49.7 ± 0.4
GalA[a]	18.0 ± 0.4	27.2 ± 0.2	14.4 ± 0.2	15.0 ± 0.4	46.7 ± 0.3	20.9 ± 0.2
GlcA[a]	12.8 ± 0.3	8.5 ± 0.1	5.3 ± 0.1	13.4 ± 0.3	13.5 ± 0.1	5.9 ± 0.1
Kdo[a]	–	16.8 ± 0.2	10.5 ± 0.1	–	36.2 ± 0.3	11.2 ± 0.1
Dha[a]	–	9.1 ± 0.1	5.7 ± 0.1	–	19.5 ± 0.2	6.1 ± 0.1
AceA[a]	–	14.2 ± 0.1	10.3 ± 0.1	–	24.0 ± 0.2	9.7 ± 0.1

[a] 2-*O*-Me Fuc, 2-*O*-methyl fucose; 2-*O*-Me Xyl, 2-*O*-methyl xylose; GalA, galacturonic acid; GlcA, glucuronic acid; Kdo, 3-deoxy octulosonic acid; Dha, 3-deoxy-D-*lyxo*-heptulosaric acid; AceA, aceric acid.
[b] <1 mg l^{-1}.

presence of AGs in the case of rhamnose, arabinose and galactose.

Fraction B3 contained all the sugars known to participate in the composition of wine polysaccharides but they were present only in small amounts (Table 6). The presence of these carbohydrates was attributed to low molecular weight arabinogalactans and mannans and oligomers of homogalacturonans and rhamnogalacturonans. Homo- and rhamnogalacturonans oligomers (GU) were estimated from the galacturonic acid content [10]. All the diagnostic sugars of RG-II were found although the presence of RG-II in this fraction has never been reported [35] and it has a higher molecular weight than the molecules eluting in this fraction (see Section 3.2). In fact, it is possible that the presence of these rare sugars was due to low molecular weight fragments of the RG-II rather than to the entire molecule. Thus, RG-II was not quantified in this fraction.

The polysaccharide content (AGs, mannans and mannoproteins, RG-IIs and galacturonans) of wine samples was deduced from the sums of the respective polysaccharides present in fractions B1, B2 and B3 (Fig. 5). The comparison of the polysaccharide contents of both wines allowed an evaluation of the action of the commercial enzymes added to the EXV wine. In both wines, the polysaccharides consisted mainly of arabinogalactans, arabinogalactan-proteins, mannans, mannoproteins and rhamnogalacturonans-II (Fig. 5). AGs represented about 40% of total soluble polysaccharides in both samples, which is in good agreement with previous observations [13,14]. However, the RG-II level found (38% in the control wine and 46% in the EXV wine) was high in comparison with previous studies [16,17], although these studies dealt with Carignan noir red wines, which present a different polysaccharide quantity than Tempranillo wines. Mannoproteins and mannans were about 19% and GUs represented only a small percent (2%).

The content of mannans and mannoproteins was very similar in both wines with values in the range of 141 (control

Table 7
Polysaccharide concentration (mg l^{-1}) of Wine fractions B1, B2 and B3

Wines	Fractions	AG[a]	M[b]	RG-II[c]	GU[d]
Control wine	B1	211	106	–	–
	B2	76	18	289	–
	B3	21	17	–	14
EXV wine	B1	297	114	–	–
	B2	120	36	544	–
	B3	28	21	–	21

[a] AG, arabinogalactans and arabinogalactan-proteins.
[b] M, mannans and mannoproteins.
[c] RG-II, rhamnogalacturonans-II.
[d] GU, oligomers of homo- and rhamnogalacturonans.

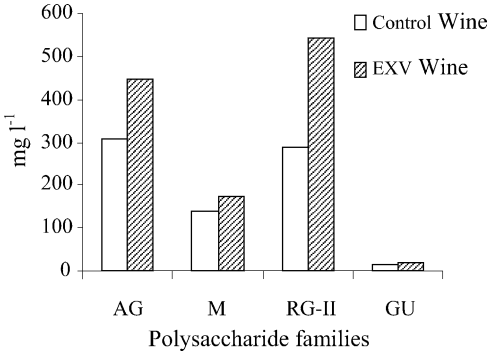

Fig. 5. Total concentration of AG, M, RG-II and GU in wines. AG, arabinogalactans and arabinogalactan-proteins; M, mannans and mannoproteins; RG-II, rhamnogalacturonans-II; GU, oligomers of homo- and rhamnogalacturonans.

wine) to 171 mg l^{-1} (EXV wine). The same commercial yeast strain was used to produce of both wines, which excluded any variability based on the nature of the strain. A completely different behaviour was observed with AGs and RG-IIs. The content of AGs was much higher in the wine treated with enzymes than in the control, and the amount of RG-II was almost double in the EXV wine. These findings indicated that solubilisation of arabinogalactans, arabinogalactan-proteins and RG-IIs had occured during the maceration–fermentation process of EXV wine due to the action of the commercial enzymes added. These enzymes hydrolysed the polysaccharides from grape berry cell walls but did not affect yeast cell wall polysaccharides.

4. Conclusions

Wine polysaccharides play an important role in wine technology, either for their sensory characteristics, their implications during fermentation or their detrimental role in filtration. Enzymic treatments represent powerful tools to control these phenomena since they may alter wine polysaccharide composition. In this study, the influence of commercial enzymes on wine polysaccharide content was analysed. Tempranillo wines were made using maceration commercial enzyme preparations together with controls.

The analytical method for the quantification of wine polysaccharides was carried out by a multistep procedure: concentration of wine, precipitation of polysaccharides by the addition of an acid-ethanol medium, fractionation of polysaccharide families by HRSEC on a Superdex-75 HR column, and determination of carbohydrate compositions of the fractions by GC-FID and GC-MS of their trimethylsilyl-ester *O*-methyl glucosides (TMS) after acidic methanolysis and derivatization. The polysaccharide content of each fraction was estimated from the concentration of individual glycosyl residues that are characteristic of well-defined wine polysaccharides.

This study indicated that wine and must samples needed to be concentrated (tree times for musts and five times for wines) before precipitation in order to ensure the quantitative precipitation of all polysaccharide families. The proposed method presented a good sensitivity, reproducibility and accuracy.

Soluble polysaccharides in wine consisted essentially of grape cell wall polysaccharides: arabinogalactans and arabinogalactan-proteins (38–41%), and rhamnogalacturonans-II (38–46%). Yeast mannans and mannoproteins were also present but in smaller amount (14–19%). In comparison with the controls, wines treated with commercial enzymes presented higher concentrations of arabinogalactans, arabinogalactan-proteins (445 mg l^{-1} versus 308 mg l^{-1}) and rhamnogalacturonans-II proteins (544 mg l^{-1} versus 289 mg l^{-1}). The content of mannans and mannoproteins was similar in both wines. These findings indicated that grape pectic polysaccharides were hydrolysed and solubilised during the maceration–fermentation due to the action of the commercial enzymes added. However, these enzymes had no influence in yeast parietal polysaccharides.

The precipitation of wine polysaccharides was observed as an important phenomenon occurring during the maceration–fermentation process, as the polysaccharide content present in the insoluble pellets accounted for a relatively significant proportion of total wine polysaccharides (29–44%).

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