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Characterization of a neutral polysaccharide with antioxidant capacity from red wine

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ABSTRACT

A neutral fraction (PS-SI) (0.3 g/L) with MW of 74 kDa, which contained galactose, arabinose, mannose, and glucose in the molar ratio of 1.0:0.6:0.4:0.2 was obtained by treatment of the whole polysaccharide extracted from red wine with cetrimide, followed by gel permeation chromatography. Spectroscopic and methylation analyses indicated that PS-SI is a mixture of neutral polysaccharides, consisting mainly of β (1→3)-linked galactopyranosyl residues, with side chains of galactopyranosyl residues at positions *O*-6. Arabinofuranosyl residues linked α (1→5), α-mannopyranosyl and glucosyl residues appear to be components of different polysaccharides. The in vitro antioxidant capacity of fractions of wine polysaccharide was studied by hydroxyl radical scavenging and ORAC assays. Fraction PS-SI presented the strongest effect on hydroxyl radicals (IC_{50} = 0.21).

1. Introduction

Polysaccharides in red wine originate either in grape berries (*Vitis vinifera*) or from yeast during fermentation. They comprise arabinogalactan proteins and rhamnogalacturonans from grape, and yeast-derived mannoproteins.[1–5] The presence and concentration of polysaccharides in wine depend on the grape variety, maturity, and climate, among numerous factors.[6,7] Wine polysaccharides play several roles such as controlling wine stability, and increasing organoleptic properties.[8–10] The antioxidant activity of red wines is well known and it has been related to the presence of phenolic compounds.[11–14]

It has been found that some polysaccharides from yeast, seaweeds, and fungi possess in vitro antioxidant activities.[15–20] Recently, Luo and Fang[21] reported the antioxidant properties of neutral glucans from ginseng. In another report, Zou et al. found that sulfation of neutral (1→3)-linked D-galactans from lac tree afforded derivatives with good antioxidant capacities.[22] As far as we know, the antioxidant capacity of wine polysaccharides has not yet been studied. In this work, we report the characterization of Cabernet Sauvignon red wine polysaccharides and the study in vitro of their antioxidant properties.

2. Results and discussion

Liquid–liquid extraction of Chilean Cabernet Sauvignon red wine followed by ethanol precipitation, gave a beige solid (PS) at a concentration of 1.6 g/L. The chemical composition (Table 1) suggested the presence of mannans, arabinogalactans, and pectin type polysaccharides. The FT-IR spectrum of the polysaccharide shows a shoulder around 1750 cm^{-1}, which was assigned to the C=O stretching vibration of an ester function, and two strong bands at 1617 and at 1067 cm^{-1}, the latter flanked by shoulders (Fig. 1A). Better characterization was achieved from the second-derivative FT-IR spectrum. Second-derivative FT-IR spectra give more information than the normal spectra, and they have been used for the characterization of polysaccharides and biomolecules present in organisms such as red seaweeds, fungi, and bacteria.[23–26] In the second-derivative spectrum of the crude polysaccharide (Fig. 1B), it can be seen that the band at 1617 cm^{-1} in the normal spectrum is resolved into three bands, two of them could be assigned to amide I and to the carboxylate group vibrations (1660.4 cm^{-1} and 1601.7 cm^{-1}, respectively).[27] Furthermore, the broad band centered around 1067 cm^{-1} in the normal spectrum is resolved into a band at 1153.1 cm^{-1}, assigned according to Kačuráková et al.[28] to arabinogalactan, and bands at 1071.7 and 976.0 cm^{-1} assigned to rhamnogalacturonan.[29] Absorptions at 881.0 and 839.2 cm^{-1} indicate the presence of β- and α-linked galactopyranosyl residues, respectively.[30] The polysaccharide was shown by gel permeation chromatography (GPC) to be composed of at least six fractions with molecular weight distribution between 70 kDa and 700 kDa (Fig. 2).

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Table 1
Chemical composition (%) of wine polysaccharides

Components	PS	PS-S	PS-SI	PS-P
Neutral Sugar[a] (%)	46.5	79.6	63.6	38.7
Galactose	1	1	1	1
Rhamnose	tr[b]	tr	tr	0.4
Arabinose	0.6	0.1	0.6	1.1
Mannose	0.6	2	0.4	0.8
Xylose	tr	tr	tr	0.1
Glucose	tr	0.5	0.2	0.1
Uronic acids (%)	32.5[c]	nd[d]	nd[d]	43.1[c]
Proteins (%)	6.1	9.9	0.5	2.1

PS—whole polysaccharide, PS-S—fraction soluble in cetrimide, PS-SI—homogeneous fraction from PS-S, PS-P—fraction insoluble in cetrimide.
[a] By GC analysis.
[b] tr = traces.
[c] By the method of Blumenkrantz and Asboe-Hansen (1973).
[d] nd = non-detected by the methods of Blumenkrantz and Asboe-Hansen (1973), and Filisetti-Cozzi and Carpita (1991).

Treatment of the whole polysaccharide with cetrimide gave a soluble fraction (PS-S) (0.9 g/L) containing galactose, arabinose, mannose, and glucose in the molar ratio 1.0:0.1:2.0:0.5 (Table 1). The main peak in its FT-IR spectrum appeared at 1074 cm^{-1}, flanked by two shoulders while in the second-derivative spectrum it was resolved into several bands that could be ascribed to arabinogalactans and mannans.[4,28] In this region C–OH deformation, CCH bending, C–O and C–C stretching vibrations occurred; each band may be due to contributions of two or more kinds of motions.[31] Furthermore, the second-derivative spectrum (Fig. 3A) shows the bands assigned to amide function of proteins at 1652.1, 1558.1, and 1419.3 cm^{-1}. Gel permeation chromatographic analysis of the cetrimide soluble fraction (PS-S) indicated the presence of at least five fractions. Only one fraction could be separated by preparative GPC, which by freeze-drying gave a white solid (PS-SI, yield 0.3 g/L). It was shown to be homogeneous by GPC (Fig. 2) with a MW of 74 kDa. The molecular weight determined by the reducing-end method corroborated the value deduced by GPC. It contained galactose, arabinose, mannose, and glucose in the molar ratio 1.0:0.6:0.4:0.2. Proteins were present in low proportions and it was devoid of uronic acids (Table 1). Its UV spectrum did not show any absorbance in the range 230–600 nm.

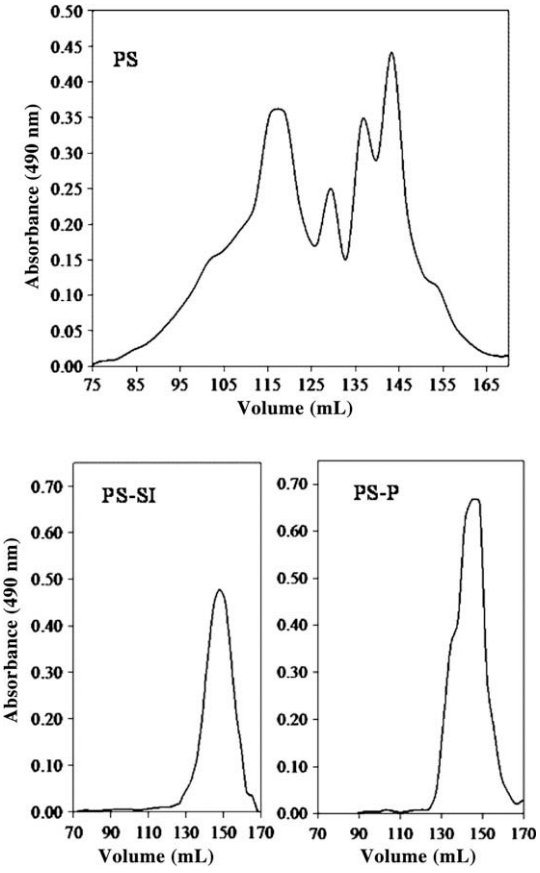

Figure 2. GPC on Sephadex 4B-CL of wine polysaccharides. PS—wine polysaccharide, PS-SI— neutral fraction from fraction soluble in cetrimide (PS-S), PS-P—fraction insoluble in cetrimide.

According to these results, the second-derivative FT-IR spectrum of PS-SI shows a weak signal assigned to amide groups and no absorption attributed to carboxyl group is present (Fig. 3B). The band at 1069.6 cm^{-1} in the normal FT-IR spectrum is resolved in the second-derivative spectrum into four signals, the peak at

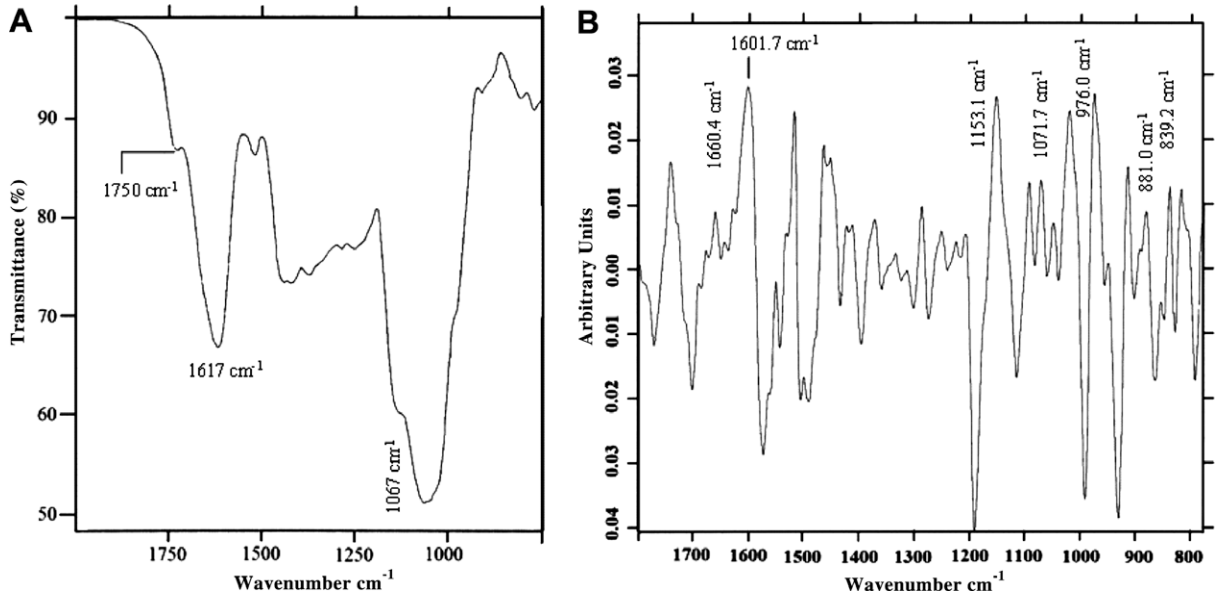

Figure 1. FT-IR spectra of the wine polysaccharide (PS), A—Normal spectrum, B—Second-derivative spectrum.

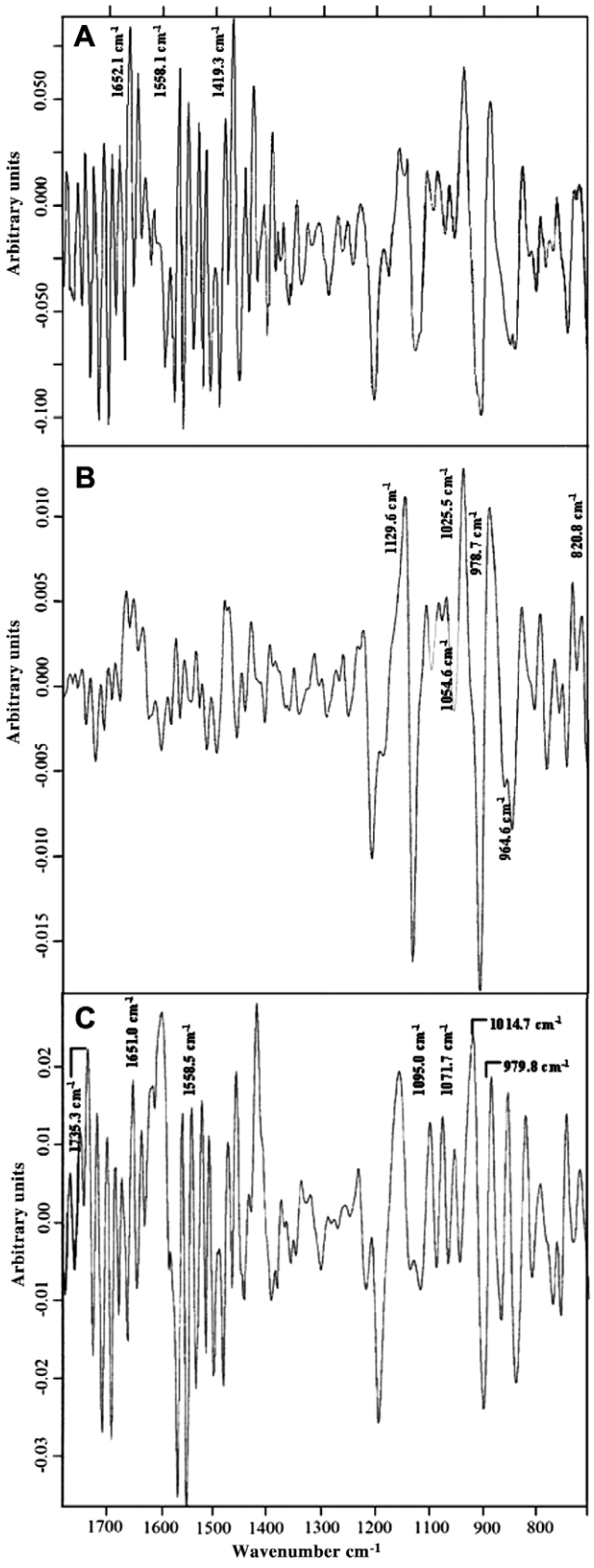

Figure 3. Second-derivative FT-IR spectra of fractions of wine polysaccharide. (A) PS-S—fraction soluble in cetrimide, (B) PS-SI—neutral fraction from PS-S, (C) PS-P—fraction insoluble in cetrimide.

1129.6 cm^{-1} was assigned together with the band at 978.7 to the presence of β-galactan-linked (1→3),[28] and the band at

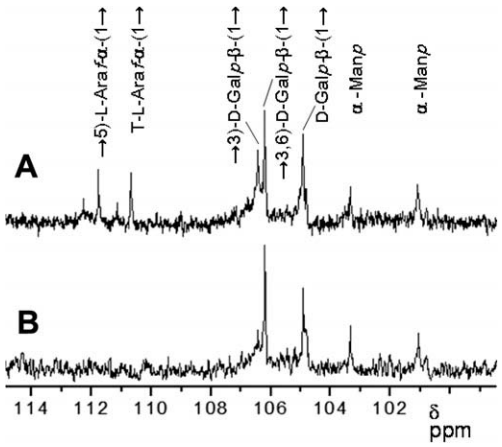

Figure 4. ^{13}C NMR spectra: (A) PS-SI (neutral fraction from PS-S), (B) partial hydrolysate of PS-SI.

1025.5 cm^{-1} could be assigned to α-linked arabinose units. The latter may be also assigned together with those at 1054.1, 964.6, and 820.8 cm^{-1} to α-linked mannosyl residues.[4]

The ^{1}H NMR spectrum of fraction PS-SI (figure not shown) was very complex, it showed in the α-anomeric region signals at 5.42 and 5.27 ppm assigned to α-L-arabinofuranosyl residues, and six bands between 5.14 and 5.04, which may be assigned to β-L-arabinopyranosyl and α-mannosyl residues. The signal at 4.47 ppm was assigned to the anomeric proton of β-D-galactopyranosyl residues. The ^{13}C NMR spectrum (Fig. 4A) of PS-SI shows several signals in the anomeric region probably due to the presence of α-linked L-arabinofuranosyl, β-linked D-galactopyranosyl, and α-linked mannopyranosyl residues.[32–35] It should be noted that the absolute configurations of monosaccharides were assigned according to the literature data on red wine polysaccharides.[36]

Signals due to the presence of α-L-arabinofuranosyl residues linked (1→5) and β-D-galactopyranosyl residues linked (1→3) and (1→3,6) were assigned with the aid of the COSY spectrum, and HSQC spectrum (Table 2, Fig. 5) and were shown to be in good agreement with those reported for arabinogalactans by Nunes et al.[34] Based on the literature, other signals in the anomeric region of the HSQC spectrum may be attributed to α-mannopyranosyl residues.[35] The ^{13}C–^{1}H HMBC spectrum was not well resolved and failed to show significant connectivities among the glycosyl residues. It should be pointed out that the ^{1}H and ^{13}C NMR spectra did not show signals in the aromatic region, which indicated the absence of phenolic contaminants.

The ^{13}C NMR spectrum of the partial hydrolyzed PS-SI (Fig. 4B) did not show the signals assigned to arabinofuranosyl residues, which may indicate that these residues were linked as branches to the galactan. The ^{1}H NMR spectrum (figure not shown) shows in the α-anomeric region six signals assigned to mannopyranosyl residues. Upon partial hydrolysis, the resolution of ^{13}C–^{1}H HMBC

Table 2
^{1}H and ^{13}C NMR chemical shifts of the most significant signals in the spectra of fraction PS-SI

		1	2	3	4	5	6
→5)-L-Araf-α-(1→	^{13}C	111.9	84.48	78.74	86.91	68.14	
	^{1}H	5.27	4.21	4.02	4.1	3.98	
→3)-D-Galp-β-(1→	^{13}C	106.8	73.3	84.55	71.73	—	64.19
	^{1}H	4.69	4.69	3.89	3.97	—	3.78,3.89
→3,6)-D-Galp-β-(1→	^{13}C	106.2	73.83	83.2	71.91	76.49	72.67
	^{1}H	4.47	3.55	3.74	4.14	3.91	4.04

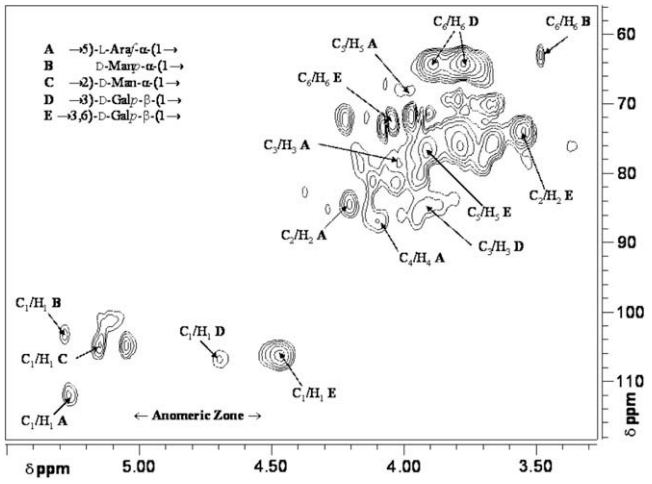

Figure 5. HSQC 2D NMR spectrum of PS-SI (neutral fraction from PS-S).

and HSQC NMR spectra of the polysaccharide did not improve, indicating the presence of different types of mannosyl residues.

The results of methylation analysis of PS-SI are given in Table 3. Arabinose was found to be involved in three types of linkages comprising 28.4% of methylated alditol acetates. According to the NMR results, probably most of the arabinose units are in furanose form, 5-linked. Also, a small amount of 3-linked arabinopyranosyl residues was present. The methylated derivatives of galactose occur in pyranose form. The most abundant galactitol derivative indicated the presence of 3,6-linked galactopyranosyl residues. The presence of 2,4,6-tri-*O*-methylgalactose derivative indicated that part of galactopyranosyl residues are linked (1→3). The mannitol derivatives indicated that mannopyranosyl residues are forming highly branched linkages. Only 1.4% of hexa-*O*-acetyl-alditol was detected, which indicated low amount of under methylation.

The results of methylation and spectroscopic analyses indicated that PS-SI mainly consists of a backbone of β-(1→3)-linked galactopyranosyl residues, substituted at *O*-6 positions with side chains of galactosyl residues. Substitution at the same position by arabinose residues could be proposed. Similar features were found in arabinogalactans and arabinogalactan–protein conjugates from different origins such as red wine, plants, and grape.[2,28,32–34,37] But, we could not find any direct evidence of the interconnections of galactose and arabinose by 2D NMR spectroscopy. Although PS-SI was homogeneous by GPC, the possibility that arabinose is part of a separate arabinan must be considered. The isolation of a linear arabinan from red wine was described by Belleville et al.[38] Furthermore, mannose and glucose must not be considered as substituents of galactan but as components of different polysaccharides. However, glucose is commonly present in low amounts in arabinan and in arabinogalactan–protein conjugates.[3,38]

Table 3
Methylation analysis of fraction PS-SI (relative mol %).

O-Methyl-alditol acetates	Linkage type	Mol %
2,3,5-Ara[a]	Terminal	7.4
2,3-Ara	1→4, 1→5	14.7
2,4-Ara	1→3	6.3
2,3,4,6-Gal	Terminal	14.7
2,3,4,6-Glu	Terminal	5.4
2,3,4,6-Man	Terminal	7.6
2,4,6-Gal	1→3	11.5
2,3,6-Man	1→4	4.7
2,4-Gal	1→3,6	19.7
3,4-Man	1→2,6	7.9

[a] 2,3,5-Ara denotes 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, etc.

The fraction insoluble in cetrimide (PS-P, yield 0.4 g/L) comprised an apparently homogeneous acidic polysaccharide by GPC (MW 80 kDa) with a low protein content. Its FT-IR spectrum shows three strong bands at 1616.1, 1419.2, and 1050.4 cm^{-1} whereas in the second-derivative spectrum these bands are resolved into more than one signal. The spectrum shows bands at 1735.3 cm^{-1} assigned to C=O stretching vibration of an ester group, two bands assigned to amide I and amide II vibrations (1651.0 and 1558.5 cm^{-1}), and four bands attributed to rhamnogalacturonan I type polysaccharide (1095.0, 1071.7, 1014.7, and 979.8 cm^{-1}),[28,29] (Fig. 3C). Furthermore, the spectrum shows the band at 1419.3 cm^{-1} assigned to C–OH deformation vibration overlapped with the O–C–O symmetric stretching vibration of carboxylate group and at 949.2 cm^{-1} assigned to C–O stretching vibration of uronic acids.[39] Fraction PS-P after total acid hydrolysis and HPLC analysis was shown to contain galacturonic acid as the uronic acid. However, the low amount of rhamnose in relation to galacturonic acid (Table 1) may indicate the presence of a polygalacturonan besides a branched rhamnogalacturonan.

From the ^{1}H and ^{13}C NMR spectra scarce information about its structure was obtained. The ^{1}H NMR spectrum (figure not shown) shows in the α-anomeric region a signal at 5.26 ppm, which was assigned to an α-D-galacturonic acid linked (1→4), and five signals between 5.23 and 4.94 ppm, which may be attributed to the presence of the same residue branched at different positions.[40] The signal at 4.73 ppm may be due to the α-anomeric proton of L-rhamnosyl residues, the presence of this residue was confirmed by the singlet at 1.17 ppm due to the methyl protons. The ^{13}C NMR spectrum is not well resolved, and shows signals at 176.74 (C_6 of galacturonic acid), eight signals in the anomeric region, which indicated highly branched complex pectic polysaccharide (Fig. 6), and at 18.73 and 18.57 assigned to C_6 of rhamnosyl residues.[40,41] From all the spectroscopic data and chemical composition, fraction PS-P, which precipitated with cetrimide, may be characterized as a mixture of complex rhamnogalacturonan and polygalaturonan.

The antioxidant capacity of the whole polysaccharide and its fractions was studied in vitro by hydroxyl radical scavenging (HRS), and ORAC assays; results are shown in Figure 7. The polysaccharides exhibited hydroxyl radical-scavenging activity in a concentration-dependent manner. The polysaccharide IC_{50} values are similar to those reported for the water-soluble neutral polysaccharides extracted from the fungus *Isaria farinosa*[17] and for the glucogalactomannan isolated from *Cordyceps militaris*.[20] The homogeneous neutral fraction PS-SI presented the strongest effect on hydroxyl radicals with an IC_{50} value of 0.21. Phenolic com-

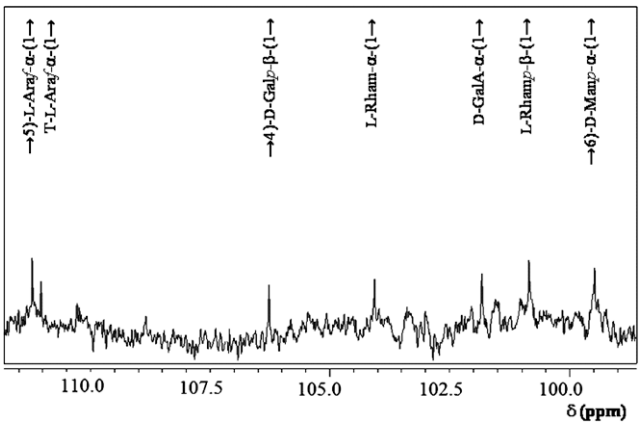

Figure 6. ^{13}C NMR spectrum of PS-P (fraction insoluble in cetrimide).

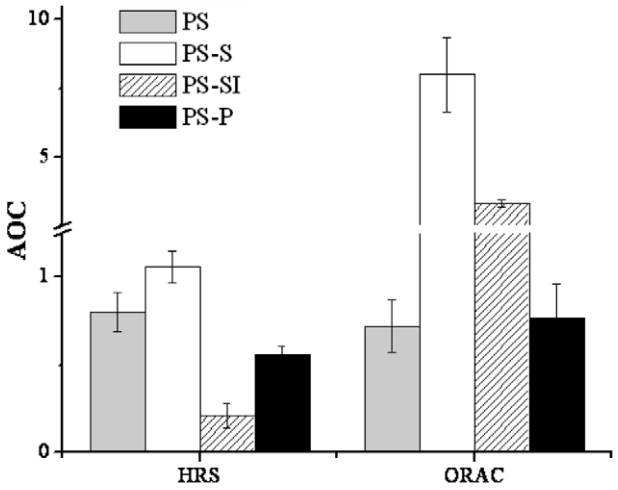

Figure 7. Antioxidant capacities of wine polysaccharides and fractions from cetrimide. Average values and standard deviation (error bars) are shown. HRS—Hydroxyl radical-scavenging activity is represented as $IC_{50,}$ this value is the amount of sample needed to inhibit free radical concentration by 50%, and is expressed in units of mg/mL. ORAC: Values represent the concentration (mg/mL) of polysaccharides solution that produces the same effect as 1 mg/mL of ascorbic acid.

pounds that could interfere in the antioxidant activity were not present as shown by UV and NMR results.

The oxygen radical absorbance capacity (ORAC) assay is based on the hydrogen atom transfer reaction, employing AAPH as free radical source; fluorescein was used as the target compound.[42] In Figure 8, the effect of fraction insoluble in cetrimide (PS-P) on fluorescein consumption induced by AAPH is presented. The areas under the curve (AUC) show a linear response related to the amount of polysaccharide. The IC_{50} of the whole polysaccharide is very similar to that of its acidic fraction, the latter presented a very complex structure with ramifications and high content of uronic acids, which may play a predominant role in its antioxidant properties. Xue et al.[43] found that alginic acid, a polyuronic acid from brown seaweed, showed antioxidant effect on a phosphatidylcholine liposomal suspension containing the radical emitter 2,2′-azobis(2-amidinopropane)-dihydrochloride. However, antioxidant capacity studies of the polysaccharides from *Fucus vesiculosus*, measured using the ferric reducing antioxidant power (FRAP) assay, indicated that the fraction rich in alginates exhibited lower antioxidant capacity than fractions containing sulfated fucans.[44] Rocha de Souza et al.[18] found similar results in the study of antioxidant properties of seaweed polysaccharides. It can be mentioned that due to the insolubility of the polysaccharides from red wine in methanol, the DPPH assay was tried in DMSO solutions. However, the assay did not show any significant activity when polysaccharides were tested in a concentration range of 0.1–10 mg/mL.

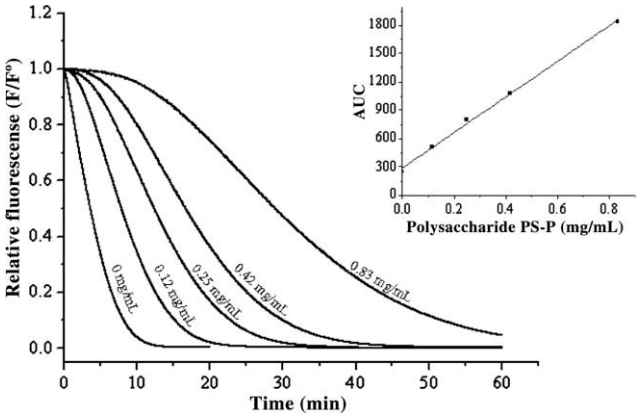

Figure 8. Effect of PS-P on fluorescein consumption induced by AAPH. Insert: dependence of the area under the curve after 80% of reaction versus fraction insoluble in cetrimide (PS-P).

It can be concluded that Cabernet Sauvignon red wine produced in the central valley of Chile contains a complex mixture of polysaccharides. Among the components characterized are a mixture of neutral polysaccharides mainly composed of a galactan, and a mixture of acidic polysaccharides. Second-derivative FT IR spectroscopy is a good technique for the characterization of wine polysaccharides in the solid state. The results of the present study indicate that wine polysaccharides exhibit antioxidant capacity, which might play a significant role in the antioxidant properties of red wine. Further studies on the antioxidant properties of other pure fractions present in red wine, such as mannan–protein fractions, are warranted.

3. Experimental

3.1. Materials and methods

Samples of Cabernet Sauvignon vintage 2005 red wine were supplied by a local producer. Unless otherwise stated all the chemicals were from Merck (Darmstadt, Germany).

3.2. Chemical analyses

Neutral sugars were determined by the phenol–sulfuric acid method using D-galactose as standard.[45] The molecular weight was determined by the reducing end method as previously described.[46] The content of uronic acid was determined following the colorimetric method of Filisetti-Cozzi and Carpita[47] and of Blumenkrantz and Asboe-Hansen[48] using D-galacturonic acid (Sigma, St. Louis, USA) as standard. Absorbance was measured in a Genesys 5 Thermospectronic spectrophotometer. Gas–liquid chromatography (GLC) was carried out on a Shimadzu GC-14B chromatograph equipped with a flame ionization detector using a SP 2330 column (0.25 mm × 30 m) and performed with an initial 5 min hold at 150 °C and then at 5 °C min^{-1} at 210 °C for 10 min. The helium flow was 20 mL min^{-1}. GLC–MS analysis was performed with an Agilent Technologies 7890A GC fitted with an Agilent 19091S-433 column (30 m × 250 μm × 0.25 μm) interfaced to an Agilent Technologies 5975C INERT XL M8D mass spectrometer. Conversion of GLC areas to molar basis was calculated for the partially methylated alditol acetates according to the effective carbon response theory of Sweet, Shapiro, and Albersheim.[49]

3.3. Spectroscopic analyses

FT-IR spectra of the samples in KBr pellets (10% w/w) were registered in the 4000–400 cm^{-1} region using a Bruker IFS 66*v* instrument.[39] Derivation including the Savitzky–Golay algorithm with 23 smoothing points was performed using the OPUS/I.R. version 1.44 software incorporated into the hardware of the instrument.

Samples of polysaccharide and its fractions in water (1 mg/mL) were analyzed by UV–vis spectroscopy in the range 230–600 nm using the Genesys 5 spectrophotometer.

^{1}H NMR (400.13 MHz) and ^{13}C (100.62 MHz) spectra of the polysaccharides were recorded in D_2O, after isotopic exchange (3 × 0.75 mL) at 70 °C on a Bruker Avance DRX 400 spectrometer using the sodium salt of 3-(trimethylsilyl)-1-propane-d_4-sulfonic acid) as internal reference. All two-dimensional experiments were acquired using a pulse field gradient incorporated into NMR pulse sequence. The two-dimensional homonuclear ^{1}H–^{1}H spectra were acquired with 128 × 2040 data points having a spectral width of

1200 Hz and processed in a 1024 × 1024 matrix to give a final resolution close to 2.3 Hz/point in the two dimensions. The two-dimensional heteronuclear single quantum coherence (HSQC) correlation spectra were acquired with 128 × 1024 data points and processed in a 1024 × 1024 matrix to give a final resolution close to 2.3 Hz/point in ^{1}H and close to 2.4 Hz/point in ^{13}C. The number of scans in each experiment was dependent on the sample concentration.

3.4. Extraction

A sample of wine (500 mL) was concentrated in vacuo at 30 °C and the residue was diluted with 1500 mL of distilled water. The aqueous solution was extracted with 2000 mL of 1-butanol, and the extraction process was repeated twice. The aqueous layers were dialyzed against distilled water using Spectra Por (MWCO 3500) membrane, water was exchanged four times. The retentate was poured over ethyl alcohol (4000 mL), and the precipitate was separated by centrifugation at 8500*g*, dissolved in distilled water, and freeze-dried, giving 0.800 g of a beige powder (PS).

3.5. Gel permeation chromatography (GPC) analysis

An aqueous solution of PS polysaccharide (1 mg/mL) was purified by chromatography on a Sepharose CL-4B column (100 × 1.5 cm). The column was calibrated with 1 mL (5 mg/mL) solutions of Blue Dextran 2000 containing D-glucose. Elution was carried out with pH 7.4 phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and monitored with the phenol–sulfuric acid reagent, and Bradford's reagent.[45,50] The column was calibrated with narrow molecular weight distribution dextrans (482, 71.327, 41.272, and 10.200 kDa (Sigma, St. Louis, USA), and dextran sulfates (500 and 8 kDa) (Sigma).

3.6. Fractionation of wine polysaccharides

Fractionation was conducted according to Scott.[51] Briefly, to a 1% solution of PS polysaccharide a 3% solution of cetrimide (*N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide) was added with stirring, until no more precipitate was formed. The mixture was stirred at 40 °C for 24 h and centrifuged at 4000*g*. The solid was dissolved in 4 M NaCl and poured into five volumes of ethanol. The resulting precipitate was dissolved in distilled water, dialyzed against distilled water, concentrated in vacuo, and freeze-dried, affording a beige solid (PS-P). To the supernatant, after the treatment with cetrimide, a 10% solution of NaI was added until no further precipitation occurred. The mixture was centrifuged, the supernatant was concentrated in vacuo, and freeze-dried. The GPC of the obtained white solid (PS-S) shows that it is composed of at least five fractions. It was submitted to fractionation by GPC on a Sepharose CL-4B column (120 × 2.5 cm) as in 3.5. Samples eluted between 480 and 576 mL were collected, dialyzed against distilled water (6 × 1000 mL), and freeze-dried (PS-SI). Aliquots (1 mg/mL) were analyzed by GPC.

3.7. Total hydrolysis

3.7.1. Hydrolysis of PS, PS-S, and PS-SI

The whole polysaccharide (PS) and fractions PS-S and PS-SI were hydrolyzed with 2 M TFA for 2 h at 120 °C and the resulting sugars were reduced with $NaBH_4$, acetylated with Ac_2O–pyridine and analyzed by GLC.

3.7.2. Hydrolysis of PS, PS-P

The acidic fraction PS-P (20 mg) was hydrolyzed with 20 mL of 90% formic acid according to the procedure described by Chandía et al.[52] The resulting syrup was dissolved in 5 mL distilled H_2O and applied to a column (30 × 2.5 cm) of DEAE Sephadex A-25 (Cl^-). The column was eluted with distilled H_2O, followed by 10% formic acid. Elution was monitored by the phenol–sulfuric acid assay. The fraction eluted with water was concentrated and the resulting neutral sugars were analyzed as in Section 3.7.1. The fraction eluted with formic acid was concentrated in vacuo, the acid was removed by repeated additions of water and evaporation, and the residue was examined by HPLC with an anionic exchange column of Whatman Partisil 10-Sax (250 × 4.6 mm) using 0.02 M KH_2PO_4 aqueous solution as eluant on a Merck-Hitachi L-6000 HPLC apparatus equipped with a L-4000A UV detector. D-Galacturonic acid and D-glucurono-6,3-lactone (Sigma) were used as standards.

3.8. Partial hydrolysis

PS-SI (100 mg) was stirred in concentrated HCl (1 mL) at 20 °C. After 15 min, the solution was poured into acetone (100 mL) and the precipitate was washed thrice with portions of acetone (2 mL) and dissolved in water. The resulting solution was dialyzed against 0.1 M sodium acetate followed by distilled water and freeze-dried.

3.9. Methylation analysis

Ten milligrams of PS-SI in dimethylsulfoxide were methylated twice using methyl iodide and NaOH according to the method of Ciucanu and Kerek.[53] The methylated polysaccharide was hydrolyzed with 2.0 M TFA for 2 h at 120 °C, and the partially methylated monosaccharides were reduced with $NaBH_4$, acetylated with Ac_2O–pyridine, and analyzed by GLC–MS.

3.10. Antioxidant assays

3.10.1. Hydroxyl radical scavenging (HRS) activity

Hydroxyl radicals were generated as published by Yu et al.[20] with some modifications. A mixture of 1 mL of 0.435 mM of Brilliant green (Carlo Erba, Milano, Italy), 2.0 mL of 0.25 mM solution of $FeSO_4$, 1 mL of H_2O_2 (0.6%), and varying concentrations of polysaccharides (0.05–10 mg/mL) was incubated for 20 min at 22 °C and the absorbance was measured at 624 nm. The hydroxyl radical scavenging activity was expressed as IC_{50}, the amount of the polysaccharide needed to inhibit free radicals concentration by 50%.

3.10.2. Oxygen radical absorbance capacity (ORAC) assay

The assay was conducted by an adaptation of the method published by Alarcón et al.[42] The consumption of fluorescein associated with its incubation with AAPH was estimated from fluorescence measurements. A reaction mixture containing 10 mM 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) (Sigma, St. Louis, USA) with or without the polysaccharide solutions with different concentrations (0.001–1.0 mg/mL) was incubated in phosphate buffer (75 mM, pH 7.4) at 37 °C. Fluorescein (23.3 nM) consumption was evaluated from the decrease in the sample fluorescence intensity (excitation: 491 nm, emission 512 nm). Fluorescence measurements were carried out using a Fluorolog-Spex 1681/0.22 m spectrofluorimeter.

Values of the intensity of fluorescence F in relation to initial value F° (F/F°) were plotted as a function of time. Integration of the area under the curve (AUC) was performed up to a time such that (F/F°) reached a value of 0.05. The area under the curve (AUC) was determined according to $AUC_{sample} = A + B \times C_{sample}$, where A *is* the intercept value, B the slope value, and C *is* the sample concentration in mg/mL. The IC_{50} values were calculated by interpolation of AUC_{sample} in the AUC of ascorbic acid.

3.11. Statistical analysis

The data obtained were means ± S.D. of three determinations, and followed by the Student's *t*-test. Differences were considered to be statistically significant if $P < 0.05$.

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