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Optimization of a method to extract polysaccharides from white grape pomace by-products

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ABSTRACT

The aim of this paper is to optimize a method to recover polysaccharides from white grape pomace (non-fermented), the main waste by-product of the food industry. Different conditions are tested and the polysaccharides extracted are analyzed by high performance size exclusion chromatography with refractive index detector (HPSEC-RID) and gas chromatography with mass detector (GC-MS). The extraction solvent did not show a significant effect on the polysaccharide extraction, acid pH yielded to higher efficiencies, and longer extraction times extracted more smaller polysaccharides ($\leq$5.4 kg mol^{-1}). The highest efficiencies were obtained with both solvents at pH 1 and 1:4 solid to liquid ratio. The optimum conditions selected (TA as solvent of extraction, 2.5 g L^{-1} solvent concentration, pH = 1, 1:4 solid to liquid ratio, and 18 h of extraction time) allow the extraction of polysaccharides rich in arabinose and galactose, rhamnogalacturonans, homogalacturonans and glucosyl polysaccharides, under efficient and food-safe conditions.

1. Introduction

Grape pomace is the main by-product of the wine industry, and include skins, pulps, seeds and stems. White grape pomace (non-fermented waste) represents 10 to 30% of the mass of crushed grapes (Muhlack et al., 2018). Polyphenols, proteins and polysaccharides are the main macromolecules detected in grape pomace. Grape and wine polyphenols have been extensively studied because of their important role in the technological and sensory properties in wines, and their numerous health benefits. Hence, several researchers have focused on developing new food technologies to recover phenolic compounds from grape by-products (Kwiatkowski et al., 2020a, 2020b). However, the extraction of polysaccharides from pomace by-products has not been explored and requires further investigation.

Polysaccharides are one of the main groups of macromolecules in grapes, musts, and wines, and originate both from grape cell walls and microorganisms acting during the winemaking. Major wine polysaccharides include polysaccharides rich in arabinose and galactose (PRAG), which originate from the pectocellulosic cell walls of grape berries; rhamnogalacturonans types I and II (RG-I and RG-II), which also arise from grape berry cell walls; and mannoproteins (MP) and glucans (GL), which are released by yeast during fermentation and during the aging of wines on lees. PRAG are composed mainly of arabinans, arabinogalactans, and arabinogalactan proteins (AGP).

Wine polysaccharides are known for their role as protective colloids. They have shown a positive effect on haze formation and tartrate salts crystallization. Some grape and yeast polysaccharides may cause problems in the clarification, being responsible of turbidity, and filter stoppages caused by membrane fouling (Sarapulova et al., 2018), but their contribution to viscosity has been disputed (Chong et al., 2019). Moreover, they are described for their influence on the fermentation flora, and their interaction with volatile compounds and other molecules responsible for wine flavor, color and foam (Martínez-Lapuente et al., 2013, 2020; Guadalupe et al., 2015). In recent years, many researchers have focused on the isolation and study of yeast mannoproteins. Different formulates of these compounds are nowadays produced by many oenological industries, and they are widely used in different stages of the winemaking and finning of white, rosé and red wines to improve their overall stability or sensory properties such as mouth-feel, aromatic profile and intensity or foam properties of sparkling wines. On the

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contrary, grape polysaccharides are not commercially available.

RG-II and AGP can enhance or inhibit tannin self-aggregation (Mateus et al., 2004; Watrelot et al., 2017) and reduce salivary protein-tannin interactions (Brandão et al., 2017, 2020), and thus affect the gustatory properties, fullness and body of wines (Vidal et al., 2004; Quijada-Morín et al., 2014). Grape AGP have also shown a protective effect against protein haze in white wines (Lankhorst et al., 2017), and RG-II molecules increase hydrogen tartrate crystallization at low concentrations and inhibit it at high concentrations (Gerbaud et al., 1996). Moreover, dimmer RG-II/boron can reduce the level of toxic cations in wines (Pérez et al., 2003), and AGP are better foam stabilizers in sparkling wines than MP (Martínez-Lapuente et al., 2018). Other authors have analyzed the potential applications, structure, and properties of starch from other fruits (Zhang et al., 2021).

Therefore, the use of grape polysaccharides as adjuvants during the winemaking or finning of wines could provide additional positive effects than those described for yeast mannoproteins. However, their extraction and isolation involve many complex steps and time-consuming methods (Apolinar-Valiente et al., 2010; Mendes et al., 2013; Hernández-Hierro et al., 2014), which has clearly limited their study and use. In fact, only a few researches are focused on the isolation of cell-wall material from grape skins to characterize grape cell wall composition and structure (Ortega-Regules et al., 2006; Apolinar-Valiente et al., 2010; Gil Cortiella & Peña-Neira, 2017), and most of them use toxic organic solvents, and are difficult and time-consuming methods with low applicability on an industrial scale.

Considering all these limitations, the aim of the present paper was to study and optimize a method for the extraction of grape polysaccharides. The extraction will be made directly from the white grape pomace (non-fermented waste) obtained during the winemaking and with different solvents and conditions. The paper aims to develop a method to extract polysaccharides from white grape waste by-products, which would imply the valorization of grape by-products.

2. Material and methods

2.1. Chemicals

All reagents were analytical grade unless otherwise stated. Standards of different monosaccharides were used to perform the calibration curves. D-(+)-Fucose > 98%, L-rhamnose monohydrate > 99%, 2-*O*-methyl D-xylose > 99%, L-(+)-arabinose > 99%, D-(+)-xylose > 99%, D-(+)-galactose > 99%, D-(+)-glucose 99.5%, D-(+)-mannose ≥ 99% and Kdo (2-keto-3-deoxy-D-*manno*-octulosonic acid) ≥ 97% were supplied by Sigma (Beerse, Belgium), and D-(+)-galacturonic acid > 93%, D-glucuronic acid ≥ 97% and myo-Inositol ≥ 98% (internal standard) were obtained from Fluka (Buch, Switzerland).

Ethanol 96% (v/v), hexane-(n) 99+ %, HPLC grade and acetyl chloride ≥ 98.0% were supplied by Merck (Darmstadt, Germany), and methanol anhydrous 99.8%, pyridine 99.5+ %, hexamethyldisilazane ≥ 99.0% and trimethylchlorosilane ≥ 98.0% were supplied by Merck (Darmstadt, Germany).

Hydrochloric acid 37% (E-507, F.C.C.) food grade was supplied by Panreac (Barcelona, Spain), while sodium hydroxide, ACS reagent, ≥97.0% was supplied by Merck (Darmstadt, Germany). HPLC-grade ammonium formate > 99.0% was supplied by Fluka (Buch, Switzerland) and miliQ water (Millipore, Molsheim, France) was used.

A pullulan calibration kit (Shodex P-82) was obtained from Waters (Barcelona, Spain). L-(+)-tartaric acid for analysis ≥ 99.5% was obtained by Merk (Darmstadt, Germany), ammonium oxalate 99.5–101.0% and absolute ethanol were purchased from Applichem GmbH (Darmstadt, Germany). All the solutions were filtered through a 0.45 μm filter before use in chromatography.

2.2. Equipments

A T-18 digital Ultra-Turrax (IKA-Werke GmbH & Co. Staufen, Germany) was used to homogenize the pomace samples. An ultrasonic bath Sonorex Digital 10P (Bandelin electronic GmbH & Co., Berlin Germany) was used to homogenize the samples during the extraction; a rotary evaporator Rotavapor R-200 (Büchi Ibérica S.L.U., Barcelona, Spain) with a water bath coupled to a vacuum pump R-200 (Büchi Ibérica S.L.U., Barcelona, Spain) was used to concentrate the samples. Samples were centrifuged using a Sorvall Lynx 4000 refrigerated centrifuge (Thermo Scientific, Barcelona, Spain). pH measurements were performed with a sensION 3 pH meter (Hach Lange GmbH Headquarter, Düsseldorf, Germany). High performance size exclusion chromatography with refractive index detector (HPSEC-RID) was performed using a modular 1100 Agilent liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with one G1311A quaternary pump, an on-line G1379A degasser, a G1316A column oven, a G1362 refractive index detector, and a G1313A automatic injector. The chromatograph was coupled to a Chemstation Agilent software. A CoolSafe freeze dryer (Scanvac, Lynge, Denmark) was used for sample lyophilization. Finally, gas chromatography–mass spectrometry (GC-MS) was performed using an Agilent Technologies 7890A (Agilent Technologies, Waldbronn, Germany) chromatograph with a Chemstation Agilent software data-processing software, equipped with a 7653B automatic injector coupled to a 5975C VL quadrupole mass detector (MS). The different monosaccharides were quantified in selected ion monitoring (SIM) mode, selecting the appropriate number of ions for each compound (*m/z*); D-galacturonic acid, L-rhamnose, L-fucose, D-galactose, D-glucose, D-mannose and D-xylose with 204 ion and D-glucuronic acid, L-arabinose, Kdo, 2-*O*-methyl-L-fucose, DHA and aceric acid with 217 ion, 2-*O*-methyl D-xylose with 146 ion, apiose with 191 and myo-inositol (internal standard) with 305 ion.

2.3. Grape pomace

White grape pomaces were obtained from Viura *Vitis vinifera* L. variety after the pressing during 2018 vintage. They had been harvested on 13th September (1.98 g/berry, 22.8 °Brix, pH 3.32, 6.54 g L^{-1} total acidity as g L^{-1} tartaric acid, 5.23 g L^{-1} of tartaric acid; 1.59 g L^{-1} of malic acid and 3,11 IPT 280 nm) from a Rioja Qualified Denomination of Origin (D.O. Ca Rioja) vineyard, and pressed in a pneumatic press (BucherVaslin XPro 8, France) to obtain juice. The grape pomace was immediately frozen at −15 °C after being pressed and stored for two months. The dry weight of the grape pomaces ranged from 35% w/w to 43% w/w of their weight, in good agreement with data described in bibliography (García-Lomillo & González-SanJosé, 2017).

2.4. Procedure for the extraction of soluble polysaccharides from grape pomace

After defrosting, the grape pomaces were homogenized using an UltraTurrax at 18,000–20,000 rpm in static conditions to achieve a total homogenization of the pomaces. Thereafter, homogenates were taken to test the different conditions for the extraction. The following variables were selected: type of extraction solvent, concentration of the extraction solvent, pH of the extraction solvent, solid to liquid ratio between homogenate and solvent, and extraction time. All the possible combinations among variables were tested. The extractions were performed in stirring conditions in a thermostatic ultrasonic bath at 22 °C and 35 kHz.

Assay of the solvent: the variables included the use of two different extraction solvents (aqueous tartaric acid and aqueous ammonium oxalate) prepared at several concentrations (2.5, 5.0 and 7.5 g L^{-1}). Each concentration was adjusted to four different pH values: 1.0, 3.5, 7.5 and no pH adjustment; the pH values of the non-adjusted solutions were between 2.6 and 2.8 for tartaric acid and 5.4 and 5.6 for ammonium oxalate. HCl 18% food grade and NaOH 1 M were used for pH

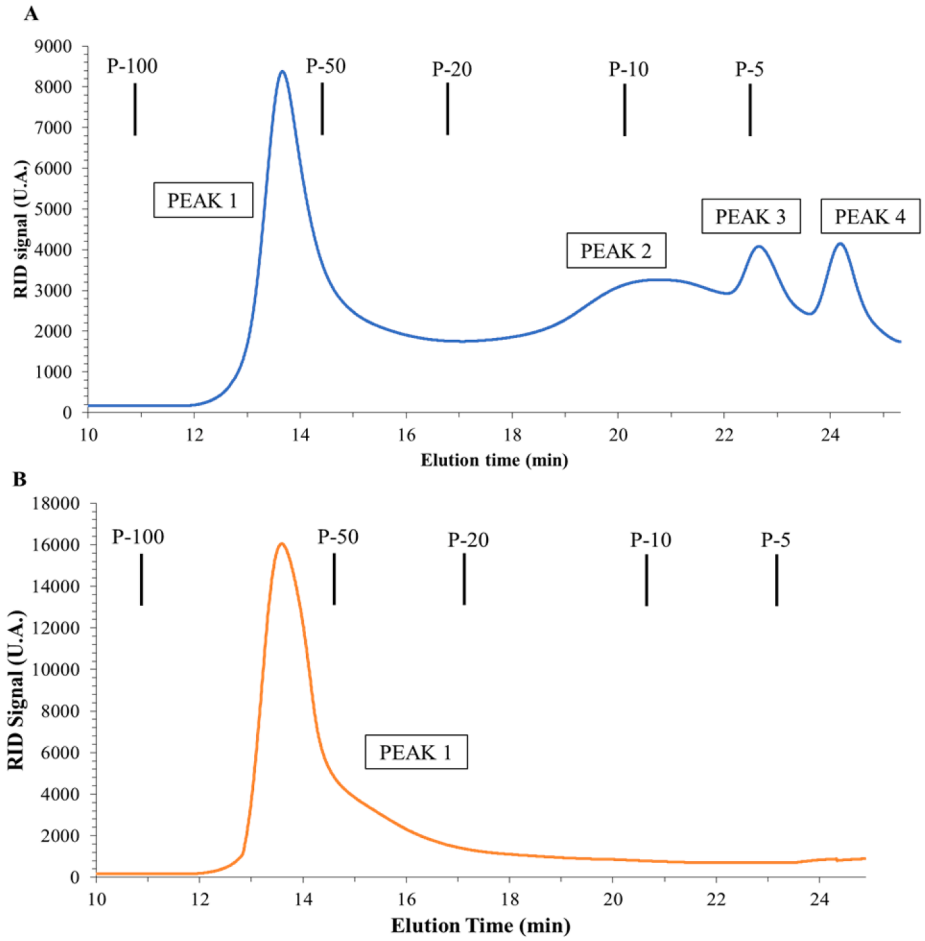

Fig. 1. HPSEC-RID chromatograms of total polysaccharides extracted from (A) ammonium oxalate (5.0 g L^{-1}, no pH adjustment, 1:4 solid to liquid ratio, 18 h of extraction time, and (B) tartaric acid (2.5 g L^{-1}, pH 1.0, 1:8 solid to liquid ratio, 4 h of extraction time). Chromatograms obtained using a Superdex 75 10/300 GL column. Elution times for the molecular weight markers (P-5 → P-50) are shown.

adjustment. Finally, 24 assays of the solvents were prepared.

Assay of solid to liquid ratio: three different solid to liquid ratio were tested between the pomace homogenates and the solvents previously prepared: 1:4, 1:8 and 1:12.

Assay of the extraction time: different times of contact between the pomace homogenates and the extraction solvents were also tested. The extractions were carried out for 1, 4 or 18 h in an ultrasonic bath. Then, the samples were centrifuged (13,600 × g for 20 min), the pellets were discarded, and the supernatants reserved for the precipitation of polysaccharides.

All the possible combinations among variables were tested, and thus 216 extraction trials were finally analysed. All the extraction trials were carried out by triplicate.

Precipitation of polysaccharides: polysaccharides were recovered in the supernatants by precipitation after sample concentration as described by Ayestarán et al. (2004) with slight modifications. The supernatants were concentrated five times with a rotary evaporator at maximum 34 °C. Total polysaccharides were then precipitated by adding four volumes of cold 96% ethanol containing 0.3 M HCl and kept for 20 h at 4 °C. Thereafter, the samples were centrifuged (33,000 × g for 20 min), the supernatants discarded, and the pellets dissolved in ultrapure water and freeze-dried. The freeze-dried precipitates contained the grape polysaccharides.

2.5. Polysaccharide analysis by HPSEC-RID

High-performance size-exclusion chromatography with a refractive index detector (HPSEC-RID) was used to obtain the molecular weights and molecular weight distributions of the grape polysaccharides. Moreover, it was also used to give an estimation of the amount of total grape polysaccharides in each assay. A Superdex-75 GL column (1.0 × 30 cm, Pharmacia, Sweden) was used at room temperature. The freeze-dried extracts were solved in miliQ water (4.0 mg mL^{-1}) and 500 μL were injected and eluted with a 0.03 M solution of ammonium formate (pH 5.8) at a flow rate of 1 mL min^{-1} (Ayestarán et al., 2004). The molecular weights and contents of the different polysaccharide fractions were determined with narrow pullulan molecular weight standards (Shodex P-82, Waters, Barcelona, Spain): P-5, Mw = 5.9 kg mol^{-1}; P-10, Mw = 11.8 kg mol^{-1}; P-20, Mw = 22.8 kg mol^{-1} and P-50, Mw = 47.3 kg mol^{-1}. The apparent molecular weights were deduced from the calibration equation log $M_w = 6.2279–0.1095\ t_R$ (t_R = column retention time at peak maximum, and $r^2 = 0.995$). Polysaccharide contents were estimated using calibration curves constructed from the pullulan P-10, P-20 and P-50, which were chosen because their peaks properly matched with those obtained for the samples. Thus, the polysaccharide content was estimated using the calibration curves $A = 3.05 \times 10E^{+06}$ g L^{-1} corresponding to P-10, $A = 3.12 \times 10E^{+06}$ g L^{-1} corresponding to P-20, and $A = 3.12 \times 10E^{+06}$ g L^{-1} corresponding to P-50 (A = peak area detected by refractive index detector of each pullulan solution, and $r^2 = 0.999$, $r^2 = 0.998$ and $r^2 = 0.998$, respectively).

The analysis of the different trials by HPSEC-RID allowed to select the ones with the highest amounts of grape polysaccharides. In these assays, the quantitation of the concrete monosaccharides and polysaccharide families was carried out by gas chromatography with mass

spectrometry detector (GC-MS).

2.6. Identification and quantitation of monosaccharides by GC-MS

The monosaccharide composition of the extracted grape polysaccharides was determined by GC-MS of their trimethylsilyl-ester *O*-methyl glycosyl-derivates (TMS) obtained after acidic methanolysis and derivatization following the methodology described by Guadalupe et al. (2012) with slight modifications. 100 μL of myo-inositol (1 mg mL^{-1}) was added to the extracts as internal standard, and freeze-dried. Thereafter, they were treated with 1 mL of the methanolysis reagent (MeOH anhydrous containing CH_3COCl 0.5 M), and the reaction was conducted in nitrogen atmosphere at 80 °C for 16 h. After removing the excess of reagent with a stream of nitrogen, the conversion of the methyl glycosides to their trimethylsilyl (TMS) derivates was performed by adding 0.5 mL of a mix of pyridine: hexamethyldisilazane: trimethylchlorosilane (10:2:1 v/v). The reaction was carried out at 80 °C for 30 min and the reagent was removed using a stream of nitrogen gas. Finally, the derivatized residues were extracted with 1 mL of hexane. GC-MS was performed with 2 μL of these solutions and the samples were analysed in triplicate. Standard carbohydrates were used as patterns for identification quantitation. The chromatographic column was an Agilent HP-5 ms fused silica GC column (30 m × 0.25 mm × 0.25 μm). The oven program started at an initial temperature of 120 °C which was increased at a rate of 1 °C min^{-1} to 145 °C and then to 180 °C at a rate of 0.9 °C min^{-1} and finally to 230 °C at 40 °C min^{-1}. The GC injectors were equipped with a 3.4 mm I.D. and were maintained at 250 °C with a 1:20 split ratio. The carrier gas was helium (99.996%) at a flow rate of 1 mL min^{-1}. Ionisation was performed by electron impact (EI) mode at 70 eV. The temperatures used were 150 °C for the MS Quad, 230 °C for the MS Source, and 250 °C for the transfer line.

The different polysaccharide families were calculated as described in Guadalupe et al. (2012). PRAG were estimated from the galactosyl, arabinosyl, rhamnosyl and glucuronosyl residues; the RG-II content was calculated from the sum of its diagnostic monosaccharides, which represent approximately 25% of the RG-II molecule. Considering the molar ratios of the RG-II (1 residue of 2-O-methyl fucose, 3.5 rhamnose, 2 arabinose, 2 galactose, 1 glucuronic acid and 9 galacturonic acid), the remaining part was attributed to the presence of AGP in the case of rhamnose, arabinose and galactose, and the remaining galacturonosyl residues was used to estimate the content of homogalacturonans (HG). Glucosyl polysaccharides (GP) were calculate from the glucose content.

2.7. Statistical analyses

All trials were made in triplicate. Statistical analyses were carried out using SPSS Statics 23 (IBM Corp., Armonk, NY, USA). In order to determine the influence of the extraction conditions, a Pearson correlation test was performed with the HPSEC-RID data. One-way analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) were applied at a confidence level of 95% (p-value of 0.05) to determine significant differences among the trials.

3. Results and discussion

3.1. Analysis of polysaccharides by HPSEC-RID

High-performance size-exclusion chromatography with refractive index detector (HPSEC-RID) was performed to obtain the molecular weight distributions and to estimate the concentration of the total grape polysaccharides extracted from the different trials. The molecular weights and amounts of the grape polysaccharides were compared to those of known pullulan standards.

Different polysaccharide profiles were obtained depending on the conditions studied. Fig. 1 shows an example of the chromatograms obtained by HPSEC after the polysaccharide extraction of the different trials. In some of the samples analyzed, the HPSEC showed the separation of grape polysaccharides in four different peaks (Fig. 1a) while in others less peaks were obtained (Fig. 1b). The refractometric profiles and the molecular weight distribution of these peaks are shown in Fig. 1.

Grape skins represent about 5 to 10% of the total dry weight of the grape berry. The composition of grape berry cell walls consists of a matrix of cellulose and associated hemicelluloses, and are particularly rich in pectin polysaccharides composed of homogalacturonans (HG), rhamnogalacturonans II (RG-II), rhamnogalacturonans I (RG-I) and polysaccharides rich in arabinose and galactose (PRAG), which comprise arabinans, arabinogalactans and arabinogalactan proteins (AGP) (Doco et al., 2007; Guadalupe et al., 2015). Celluloses and hemicelluloses, which consist mainly of xyloglucans in grape cell walls (Doco et al., 2003; Pinelo et al., 2006), are major in the skin cell wall of grape berries (Vidal et al., 2001).

As shown in Fig. 1, Peak 1 corresponded to higher-molecular-weight polysaccharides with a molecular mass higher than 40 kg mol^{-1} and an average molecular weight of 55 kg mol^{-1}. According to bibliography (Ayestarán et al., 2004; Guadalupe & Ayestarán, 2007), these molecules correspond with polysaccharides rich in arabinose and galactose (PRAG) and could also be attributed to grape structural glucosyl polysaccharides as celluloses and hemicelluloses. PRAG are the major wine polysaccharide family originated from the grapes and show a molar mass between 50 and 260 kg mol^{-1} in musts and wines (Guadalupe et al., 2015). Cellulose microfibrils are composed of α-(1–4)-linked D-glucose and represent the major constituent of the cell wall polysaccharides (Pinelo et al., 2006). Hemicellulosic polysaccharides consist mainly on xyloglucans based on backbone of β-(1–4) D-glucan with side chains containing xylose, galactose and fucose.

A second peak (Peak 2) was obtained around 20 min and showed a molecular weight between 8.4 and 16.3 kg mol^{-1} and an average molecular weight of 12.7 (Fig. 1A). According to previously published data (Ayestarán et al., 2004; Guadalupe & Ayestarán, 2007), this signal would correspond to a complex mixture of mainly RG-II dimmers, with an average molecular weight of 10–12 kg mol^{-1} (Doco et al., 1997; Pérez et al., 2003), and fragments from the PRAG and grape structural glucosyl polysaccharides. RG-II consists of a short (1–4)-α-D-galacturonan backbone branched with four different chains containing mainly rhamnose, arabinose and also some rare carbohydrates as 2-*O*-methyl-fucose, apiose, 2-*O*-methyl-xylose, Kdo (2-keto-3-deoxy-D-*manno*-octulosonic acid), DHA (3-deoxy-D-lyxo-hepyulosaric acid) and aceric acid (3-C-carboxy-5-deoxy-L-xylose); and its content in skin tissue is three-fold higher than that on pulp tissue (Vidal et al., 2001; Arnous & Meyer, 2009). RG-II exists in the cell wall as their monomer (Mw ≈ 5 kg mol^{-1}) or dimer (Mw ≈ 10 kg mol^{-1}) form (Kassara et al., 2019) that is cross-linked by a borate di-ester, and in wines is mainly found in the form of dimmers (Pérez et al., 2003).

Peak 3, with an average molecular weight of 5.4 kg mol^{-1} (Fig. 1A), would thus correspond to the monomeric form of the RG-II (Muszyński et al., 2015) and also to smaller fragments of PRAG and grape structural glucosyl polysaccharides.

Finally, Peak 4, with an average molecular weight of 3.6 kg mol^{-1} (Fig. 1A), was attributed to small oligosaccharides. In grape skin cell walls, these oligosaccharides mainly correspond to xyloglucan oligosaccharides from the exocarp and mesocarp of the cell wall material of grape berries (Doco et al., 2003), and oligomers of homogalacturonans. Homogalacturonans, which are linear chains of α-(1–4)-linked D-galacturonic acid, are also major pectic polysaccharides in grapes. RG-I molecules, with an average molecular weight around 40 kg mol^{-1}, are also abundant in grape berries; however, these polysaccharides are not soluble and difficult to extract (Vidal et al., 2001).

3.2. Effect of the extraction variables on the polysaccharide contents estimated by HPSEC-RID

Different analyses were carried out to know how the method

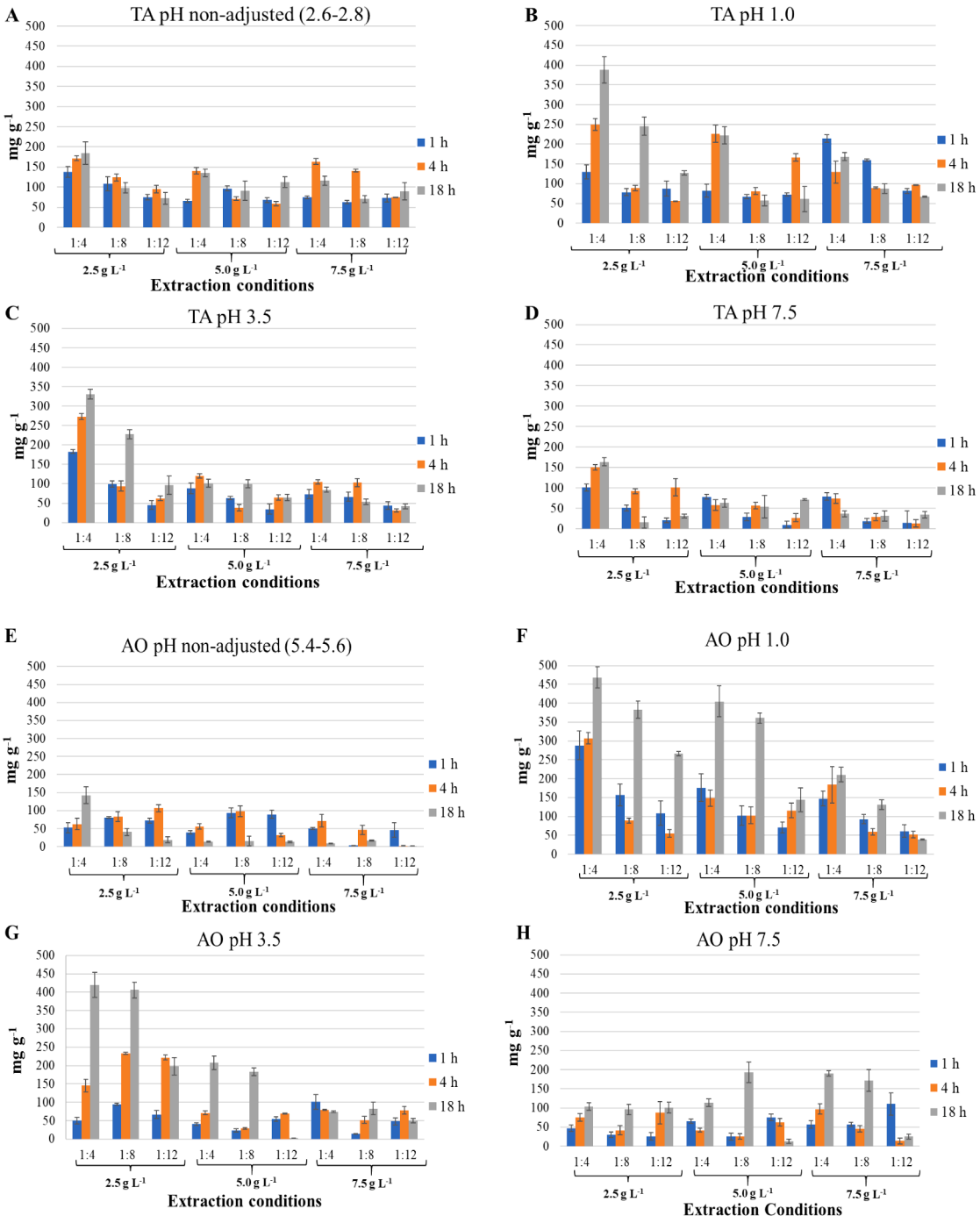

Fig. 2. Effect of the different variables on the extraction as mg of total polysaccharides per g of extract (n = 216). Total polysaccharides estimated by HPSEC-RID on a Superdex 75 10/300 GL column. A: tartaric acid with no pH adjustment (pH = 2.6–2.8); B: tartaric acid at pH 1.0; C: tartaric acid at pH 3.5; D: tartaric acid at pH 7.5; E: ammonium oxalate with no pH adjustment (pH = 5.4–5.6); F: ammonium oxalate at pH 1.0; G: ammonium oxalate at pH 3.5; H: ammonium oxalate at pH 7.5. The different solvent concentrations (2.5; 5.0 and 7.5 g L^{-1}), solid to liquid ratios (1:4, 1:8 and 1:12) and extraction times (1, 4 and 18 h) are shown. Each trial was performed in triplicate. The bars in the picture represent the standard deviation of each mean.

variables (type of solvent, pH, solvent concentration, solid to liquid ratio and time of extraction) affected the extraction of polysaccharides from the grape pomace. Fig. 2 shows the effect of the different variables on the extraction as mg of total polysaccharides estimated by HPSEC-RID per g of extract. Moreover, the HPSEC-RID was also used to estimate the amount of polysaccharides of different molecular weights, i.e., polysaccharides with an average *Mw* of 55 kg mol^{-1} (peak 1), polysaccharides with an average *Mw* of 12.7 kg mol^{-1} (peak 2), polysaccharides with an average *Mw* of 5.4 kg mol^{-1} (peak 3), and polysaccharides with an average *Mw* of 3.6 kg mol^{-1} (peak 4). A Pearson correlation analysis was carried out to determine which extraction conditions enhanced the extraction of total polysaccharides (ETP) and the polysaccharide fractions of different molecular weights (peaks 1 to 4). Result of the analysis is shown in Table 1. The correlation analysis was applied to all the data, independent of the solvent used (216 trials), and also to the data grouped by the extraction solvent (108 trials for tartaric acid and 108 for ammonium oxalate). The different variables were treated as independent factors for the correlation analysis.

Table 1
Pearson correlation data among the extraction variables and the content of total polysaccharides (ETP) and polysaccharides of peaks 1, 2, 3 estimated by HPSEC-RID.

	Extraction variables[b]	Correlation parameters[c]	ETP[d]	PEAK 1[d]	PEAK 2[d]	PEAK 3[d]	PEAK 4[d]
A[a]	**SC**	**ρ Pearson**	−0.131	−0.163	−0.022	−0.041	0.035
		***p*-value**	0.054	0.057	0.746	0.544	0.610
	pH	**ρ Pearson**	−0.341**	−0.325**	−0.115	0.389**	0.363**
		***p*-value**	0.009	0.007	0.093	0.004	0.000
	SL	**ρ Pearson**	0.087	0.131	−0.017	−0.034	0.018
		***p*-value**	0.202	0.054	0.804	0.620	0.793
	ET	**ρ Pearson**	0.177**	0.055	0.279	0.235**	0.240**
		***p*-value**	0.009	0.417	0.079	0.007	0.004
B[a]	**SC**	**ρ Pearson**	−0.197	−0.194	0.023	−0.010	−0.062
		***p*-value**	0.141	0.144	0.361	0.916	0.522
	pH	**ρ Pearson**	−0.417*	−0.473*	0.036	0.609**	0.581**
		***p*-value**	0.026	0.038	0.243	0.000	0.000
	SL	**ρ Pearson**	0.234**	0.226**	0.172	0.146	0.175
		***p*-value**	0.005	0.009	0.098	0.133	0.070
	ET	**ρ Pearson**	−0.069	−0.064	0.071	0.141*	0.124*
		***p*-value**	0.477	0.514	0.103	0.030	0.020
C[a]	**SC**	**ρ Pearson**	−0.078	−0.127	−0.032	−0.059	0.079
		***p*-value**	0.424	0.192	0.740	0.546	0.416
	pH	**ρ Pearson**	−0.244*	−0.216*	−0.225*	0.222**	0.147
		***p*-value**	0.011	0.025	0.019	0.008	0.130
	SL	**ρ Pearson**	−0.035	−0.023	−0.025	−0.066	−0.044
		***p*-value**	0.723	0.814	0.799	0.498	0.650
	ET	**ρ Pearson**	0.386**	−0.164	0.407**	0.184*	0.278*
		***p*-value**	0.000	0.091	0.000	0.006	0.036

[a] A: extracts obtained in triplicate with both solvents (n = 648); B: extracts obtained in triplicate with tartaric acid (n = 324); C: extracts obtained in triplicate with ammonium oxalate (n = 324).
[b] Extraction variables (SC: solvent concentration, S/L: solid to liquid ratio, EC: extraction time).
[c] Correlation parameters. Level of significance * and ** indicates significance at $p < 0.05$ and $p < 0.01$ respectively.
[d] ETP: Estimated total polysaccharides as the sum of polysaccharides of different molecular mass (peak 1 + peak 2 + peak 3 + peak 4); Peak 1: amount of polysaccharides with an average molecular weight of 55 kg mol^{-1}; Peak 2: amount of polysaccharides with an average molecular weight of 12.7 kg mol^{-1}; Peak 3: amount of polysaccharides with an average molecular weight of 5.4 kg mol^{-1}; Peak 4: amount of polysaccharides with an average molecular weight of 3.6 kg mol^{-1}.

The variables that presented a correlation showed a level of significance lower than $p < 0.01$ in most of the cases. Since the effect of the solvent used to obtain the extracts is a categoric variable, a correlation analysis was not appropriated to this evaluation. A one-way analysis of variance (ANOVA) was applied to determine significant differences among the trials regarding the solvent used. The effect of the extraction solvent on the extraction of polysaccharides from the white grape pomace is shown in the following subsections.

3.2.1. *Effect of the extraction solvent on the polysaccharide extraction*

It is known that polysaccharides are some of the soluble macromolecules of the cell wall grape skins, so they can be easily found in musts after pressing the grapes (Vidal et al., 2003; Guadalupe & Ayestarán, 2007). Therefore, an aqueous solution was chosen for the extraction of the grape polysaccharides. Two chelating agents were evaluated as extraction solvents, tartaric acid (TA), and ammonium oxalate (AO), in order to achieve the maximum extraction of polysaccharide families from the white grape pomace obtained after pressing. Tartaric acid was chosen because it is naturally found in grapes and musts and could contribute to a higher extraction of polysaccharides by disrupting the Ca-bridges between pectin chains (Jarvis, 2011). On the other hand, ammonium oxalate was selected as one of the most widely used chelating agents for pectin extraction. Both chelating agents had been previously tested for grape polysaccharide extraction by Gil Cortiella and Peña-Neira (2017), who observed a higher extraction of total polysaccharides with the use of ammonium oxalate than with tartaric acid. However, it is important to point out that the authors use the grape skins after destemming and peeling the grapes, and they give an estimation of the total polysaccharides by HPSEC but they do not quantify the specific grape polysaccharide families. Moreover, the use of this chelating agent was not proved with the conditions described in the present paper. Hence, and taking into account that tartaric acid is a food-safe and a food-grade reagent, and its addition is permitted in the oenological practices, we decided to test its efficiency in the present study.

Firstly, white grape pomace obtained after pressing was immediately frozen at −15 °C because previous researchers had proved that freezing the grape skins allowed an isolation of the cell wall material from the grape skins minimizing the fragmentation of the cell wall polysaccharides by endogenous enzymes (Apolinar-Valiente et al., 2010). After defrosting, the grapes were properly homogenized and the different conditions for the extraction were proved. All the possible combinations among variables were tested. Fig. 2 shows the effect of the different variables on the extraction as mg of total polysaccharides obtained by HPSEC per g of extract. All the data obtained with both solvents in the same conditions were compared, and both solvents showed similar effectiveness for the extraction of total polysaccharides. Therefore, the amount of total polysaccharides was higher with aqueous tartaric acid in 82 of the 216 trials, while this amount was higher with aqueous ammonium oxalate in 86 trials, and the rest did not show differences. These results contrasted with those of Gil Cortiella and Peña-Neira (2017), who obtained higher extraction of total polysaccharides with ammonium oxalate but used peeled skins instead of grape pomace.

When the ANOVA analysis was applied, no significant differences were found in the amount of total polysaccharides between TA and AO extracts (p-value = 0.496). In contrast, the use of the use of ammonium oxalate favored the extraction of polysaccharides of lower molecular mass (peaks 2 and 3) as significant differences were found for peak 2 (p-value = 0.00) and peak 3 (p-value = 0.00). The results of the present paper showed that the use of AO enhanced the extraction of peaks 2 and 3, which contains RG-II and HG, when high pH values (non-adjusted and 7.5) were applied (data not shown).

Both TA and AO solvens were chosen for their potential as chelators of Ca^{2+}. In the homogalacturonan polysaccharide (HG), its unmethylated galaturonic residues are negatively charged and linked with Ca^{2+} to other polysaccharides of the matrix in the plant cell walls (Goulao et al., 2012). Moreover, the dimeric form of the RG-II linked by borate diesters is also stabilized by the presence of calcium (Goulao et al.,

2012). Our results indicated that AO would cause a higher disruption of the Ca-bridges when higher pH values were used. It is important to notice that the non-adjusted pH for AO is 5.5 and it is 2.7 for TA. Moreover, the chelating activity of the TA is produced in the form of tartrate, which is predominant only at pH values higher than 4.

3.2.2. Effect of the concentration of the extraction solvent on the polysaccharide extraction

Both extraction solutions were prepared at the following concentrations, 2.5, 5.0 and 7.5 g L^{-1}. These concentrations were selected according to the contents of tartaric acid usually found in grape juices and wines (2.5–5.0 g L^{-1}). Higher concentrations (7.5 g L^{-1}) were tested to evaluate if they achieved higher extraction yields. The same concentrations were tested for aqueous ammonium oxalate.

Fig. 2 shows the mg of total polysaccharides per g of extract in relation to the extraction conditions. The results obtained with the three different solvent concentrations were compared for each solvent. With the aqueous solution of tartaric acid, 36 extracts showed higher polysaccharide concentrations with solvent concentration at 2.5 g L^{-1}, 15 extracts showed higher polysaccharide amounts with 5 g L^{-1} solvent concentration, 24 with 7.5 g L^{-1}, and 33 did not show differences. With the aqueous solution of ammonium oxalate, 30 extracts showed higher polysaccharide concentrations with 2.5 g L^{-1} solvent concentration, 24 samples showed higher polysaccharide with 5 g L^{-1}, 12 with 7.5 g L^{-1}, and 42 did not show differences. In the correlation analysis (Table 1), no correlations were obtained between the solvent concentration and the amount of total polysaccharides or polysaccharides of different molecular weights (peaks 1, 2, 3 and 4).

These results indicated that the concentration of the solvent did not affect the extraction yield of grape polysaccharides. The lowest solvent concentrations were selected to reduce the amount of reagent used.

3.2.3. Effect of the pH of the solvent on the polysaccharide extraction

Four different pH values were tested for each solvent and concentration. pH value of 3.5 was chosen as it is the pH found in musts and wines; pH 7.0 was chosen as it achieved higher extractions of polysaccharides in the study of Gil Cortiella and Peña-Neira, 2017; pH value of 1 was chosen as a physical disruption method to increase the breakdown of the grape berry cell wall. These pH values were also compared with trials with no pH adjustment.

Contrary to the type of solvent and solvent concentration, the pH of the solvent affected the extraction of total grape polysaccharides (Fig. 2, Table 1). With aqueous tartaric acid, 44 extracts showed higher polysaccharide concentrations at pH 1, 4 at non-adjusted pH, 4 at pH 3.5, 12 at pH 7.5 and 44 did not show differences. With the aqueous solution of ammonium oxalate, 45 samples showed higher polysaccharide concentrations at pH 1, 16 at non-adjusted, 16 at pH 3.5; 12 at pH 7.5, and 19 did not show differences (Fig. 2). The correlation analysis (Table 1) confirmed that acid pH increased the extraction of total grape polysaccharides quantified by HPSEC. Hence, a negative correlation was found between pH and total polysaccharides in all the analyses (for all data, for trials with TA and for trials with AO), indicating that low pH values enhanced the extraction of total polysaccharides. The same results were observed regarding the extraction of polysaccharides of high and medium molecular weight (peak 1 for TA and peak 1 and 2 for AO), indicating that acid pH favored the extraction of these polysaccharides. On the contrary, the extraction of lower molecular weight polysaccharides (peaks 3 and 4) was correlated with high pH values (positive correlation), indicating that high pH values could enhance the extraction of polysaccharide fractions of lower molecular mass.

The extraction and solubility of compounds is generally influenced by the pH, being more effective in acid conditions (Núñez-López et al., 2008), and explaining why the extraction of polysaccharides is higher in acidic conditions. As expalined above, the extraction of peaks 3 and 4 would be favoured at neutral pH due to the chelating activity of the AO and the tartrate salts on the Ca-bridges of the HG molecules.

3.2.4. Effect of the solid to liquid ratio on the polysaccharide extraction

Three different solid to liquid (S/L) ratios were tested during the extraction. The grape pomace homogenates were mixed with the solvents according to the following ratios, 1:4, 1:8 and 1:12. Grape skins represent between 8 and 10% of the mass of the grape, and thus the 1:12 ratio was selected. Higher ratios were also tested to reduce the amount of solvent used during the extraction.

It was observed that the highest S/L ratio (1:4 ratio) enhanced the extraction of total polysaccharides with the aqueous solvent of tartaric acid (Fig. 2). With tartaric acid, 57 samples showed higher polysaccharide concentrations using 1:4 S/L ratios, 12 with 1:8 S/L ratios, 0 with 1:12, and 39 did not show differences. However, with aqueous ammonium oxalate, the S/L ratio did not show a clear effect on the extraction of grape polysaccharides. Therefore, 24 samples showed higher polysaccharide concentrations at 1:4 S/L ratios, 30 at 1:8, 33 at 1:12, and 21 did not show differences.

When the analysis of correlation was carried out to all data, no correlations were found between the S/L and the amount of polysaccharides extracted (Table 1). For AO extracts, there were neither correlations between S/L and the polysaccharide extraction (Table 1). However, with aqueous tartaric acid, a positive correlation was found between S/L and total polysaccharides and peak 1, indicated that higher solid to liquid ratios (1:4) enhanced the extraction of these compounds.

3.2.5. Effect of the extraction time on the polysaccharide extraction

The last condition studied was the time of extraction. All the trials described in the previous sections were tested at different extraction times. Three different times of contact between the pomace homogenates and the solvents were analyzed: 1, 4 and 18 h (Fig. 2). Short extraction times (1 h) are usually employed in other procedures described in bibliography (Apolinar-Valiente et al, 2010). Higher times were also tested because Gil Cortiella and Peña-Neira, 2017 describe that some polysaccharides are not immediately released from the cell wall matrix, and longer extraction times (18 h) are needed to solubilize these molecules.

The relation of the extraction time and the yield of polysaccharide extraction was not clear for tartaric acid (Fig. 2 and Table 1). Therefore, with aqueous tartaric acid, 9 samples showed higher total polysaccharide concentrations with 1 h of extraction, 36 samples showed higher polysaccharide concentrations with 4 h, 29 with 18 h and 39 did not show differences (Fig. 2). For TA, no correlations were found between the extraction time and the extraction of total polysaccharides and high and medium molecular weight polysaccharides (peaks 1 and 2); only smaller fragments of polysaccharides (peaks 3 and 4) were positively correlated with the extraction time (Table 1). On the contrary, increasing the extraction time with ammonium oxalate increased the extraction of total polysaccharides from the grape pomaces. With aqueous ammonium oxalate, 18 samples showed higher polysaccharide concentrations with 1 h of extraction while 21 samples showed higher polysaccharide concentrations with 4 h and 54 with 18 h (Fig. 2). Positive correlations were obtained between the extraction time and all types of polysaccharides for ammonium oxalate, in good agreement with the results obtained by Gil Cortiella and Peña-Neira (2017).

With both solvents, increasing the extraction time significantly increased the content of polysaccharides of lower molecular weights, indicating that longer extraction times were needed to release the lower molecular weight polysaccharides.

3.3. Effect of the extraction variables on the monosaccharide and polysaccharide contents estimated by GC-MS

The analyses of the different trials by HPSEC-RID was used to select the variables enhancing the extraction of grape pomace polysaccharides. In the selected trials, a further analysis was made by GC-MS to quantify the concrete monosaccharides and polysaccharide families.

The HPSEC analyses showed that both solvents achieved similar

Table 2
Monosaccharide composition (mg carbohydrates g^{-1} of grape extract)[a] of twelve grape extracts determined by GC-MS.

Solvent[b]	pH[b]	S/L[b]	2-*O*-Me Fuc[c]	2-*O*-Me Xyl[c]	Arabinose	Rhamnose	Fucose	Xylose	Mannose	Galactose	GalA[c]	Glucose	GlcA[c]	TC[d]
TA	pH 1	1:4	0.98 (0.229) β	0.21 (0.007) β	10.4 (4.43) α	21.1 (6.28) β	0.58 (0.181) β	**11.7 (2.71)** γ	18.7 (5.70) β	**113.8 (8.95)** γ	99.0 (4.22)c	175.2 (16.47) β	**6.6 (2.12)** β	**458.3 (21.74)** γ
TA	pH 1	1:8	1.11 (0.281) β	**0.10 (0.027)** α	6.4 (2.18) αβ	**8.6 (1.03)** α	**0.29 (0.083)** α	7.5 (1.05) β	10.1 (2.34) α	66.3 (5.97) β	**43.9 (0.58)** β	145.5 (55.14) β	5.4 (1.76) β	**295.3 (55.61)** β
TA	pH 1	1:12	**0.37 (0.037)** α	**0.08 (0.016)** α	**3.0 (1.04)** α	7.4 (3.15) α	**0.17 (0.069)** α	**4.5 (1.90)** α	**7.4 (1.42)** α	29.8 (5.39) α	15.6 (3.70) α	**58.7 (29.92)** α	2.1 (0.55) α	**129.2 (30.90)** α
TA	pH 3.5	1:4	**0.83 (0.071)** Δ	0.64 (0.071) Δ	**47.3 (4.11)** Σ	**17.3 (2.34)** Σ	0.72 (0.037) Δ	**7.0 (1.09)** Σ	**23.3 (3.45)** Δ	**70.0 (2.06)** Σ	**34.0 (1.01)** Σ	171.8 (36.79) Δ	**5.8 (0.05)** Σ	378.7 (37.34) Σ
TA	pH 3.5	1:8	**0.90 (0.064)** Δ	**0.16 (0.038)** Γ	**22.1 (2.39)** Δ	**11.8 (0.29)** Δ	0.82 (0.042) Δ	4.2 (0.90) Δ	**21.1 (8.47)** Δ	53.2 (5.11) Δ	27.9 (4.43) Δ	**148.9 (19.79)** Δ	**4.1 (1.06)** Δ	**295.7 (22.74)** Δ
TA	pH 3.5	1:12	**0.35 (0.036)** Γ	**0.13 (0.022)** Γ	8.8 (2.74) Γ	**4.5 (0.74)** Γ	0.22 (0.031) Γ	2.4 (0.85) Γ	**9.1 (3.49)** Γ	**23.0 (1.74)** Γ	21.1 (3.04) Γ	**19.8 (8.22)** Γ	1.9 (0.18) Γ	**91.2 (10.04)** Γ
		pH (%)	4.26	20.46	41.09	1.00	20.16	29.35	17.69	12.47	20.45	1.10	3.92	2.22
		p-value	0.031	0.000	0.000	0.260	0.001	0.000	0.003	0.000	0.000	0.242	0.088	0.007
		S/L (%)	85.58	56.91	39.15	83.69	51.82	59.24	58.24	79.22	51.10	87.94	80.95	93.71
		p-value	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		pH × S/L (%)	1.57	21.29	18.28	6.67	15.29	3.02	8.04	7.22	28.01	2.24	1.52	1.56
		p-value	0.366	0.000	0.000	0.032	0.009	0.158	0.087	0.000	0.000	0.254	0.530	0.056
AO	pH 1	1:4	1.09 (0.220) a	0.25 (0.101) a	7.8 (2.13) b	28.7 (3.91) b	0.50 (0.187) a	**17.5 (1.44)** c	24.4 (7.43) c	**167.7 (11.11)** c	84.7 (14.31) b	172.9 (16.37) a	**15.9 (2.96)** b	**521.4 (26.12)** c
AO	pH 1	1:8	0.90 (0.113) a	**0.61 (0.110)** b	5.0 (0.84) a	**29.4 (7.30)** b	**0.42 (0.051)** a	7.2 (0.11) b	15.2 (4.30) b	73.8 (11.79) b	**102.4 (15.48)** c	170.3 (54.84) a	5.1 (0.43) a	**420.3 (58.81)** b
AO	pH 1	1:12	**1.17 (0.309)** a	**0.23 (0.078)** a	**8.4 (0.30)** b	5.5 (1.58) a	**0.47 (0.049)** a	**1.7 (0.37)** a	**3.7 (0.94)** a	22.9 (4.16) a	15.4 (3.93) a	**169.6 (8.98)** a	3.2 (1.52) a	**232.2 (10.93)** c
AO	pH 3.5	1:4	**1.78 (0.145)** B	0.45 (0.456) A	**11.2 (8.15)** A	**6.6 (5.91)** A	0.44 (0.330) A	**2.4 (2.38)** A	**5.6 (5.50)** A	**105.0 (18.36)** B	**21.5 (5.28)** A	157.4 (9.51) A	**2.5 (2.03)** A	314.8 (24.44) B
AO	pH 3.5	1:8	**1.89 (0.362)** B	**0.42 (0.086)** A	**13.9 (3.49)** A	**8.1 (1.25)** A	0.39 (0.074) A	3.8 (0.68) A	**6.6 (0.83)** A	48.3 (0.57) A	24.5 (6.01) A	**203.9 (8.78)** B	**2.8 (0.41)** A	**314.6 (11.35)** B
AO	pH 3.5	1:12	**0.99 (0.107)** A	**0.39 (0.072)** A	11.3 (1.36) A	**6.9 (0.81)** A	0.29 (0.110) A	1.8 (0.14) A	**4.4 (1.34)** A	**35.9 (3.84)** A	22.5 (2.56) A	**145.4 (13.05)** A	2.1 (0.38) A	**232.0 (14.01)** A
		pH (%)	37.42	2.61	49.34	41.88	14.67	29.69	32.08	6.42	40.43	0.15	32.24	23.99
		p-value	0.000	0.428	0.002	0.000	0.132	0.000	0.000	0.000	0.000	0.859	0.000	0.000
		S/L (%)	14.93	24.93	0.22	27.43	9.93	35.44	32.77	82.37	29.30	22.72	32.95	54.47
		p-value	0.005	0.076	0.965	0.000	0.437	0.000	0.000	0.000	0.000	0.120	0.000	0.000
		pH × S/L (%)	36.97	25.84	13.93	25.54	8.18	33.39	25.56	9.77	27.88	23.40	31.25	15.68
		p-value	0.000	0.071	0.144	0.000	0.502	0.000	0.000	0.000	0.000	0.114	0.000	0.000

[a] Values are means with their standard deviations in parentheses (n = 3). Bold font in the same column indicate statistically significant differences ($p < 0.05$) between the extraction solvent (TA and AO) with the same pH and S/L. Different letters in the same column indicate statistically significant differences ($p < 0.05$). Greek lower-case letters compare separately S/L for pH 1 for TA solvent. Greek upper-case letters compare separately S/L for pH 3.5 for TA solvent. Latin lower-case letters compare separately S/L for pH 1 for AO solvent. Latin upper-case letters compare separately S/L for pH 3.5 for AO solvent. A one-way analysis of variance (ANOVA) was used to compare the effect of the extraction solvent, and a two-way analysis of variance (MANOVA) was used to compare the effect of the pH and the S/L. A Duncan post-hoc test was used to determine significant differences. Italic font indicates the results of the MANOVA analysis and percentage of attributable variance (%).

[b] Solvent (TA: aqueous tartaric acid 2.5 g L^{-1}, AO: aqueous ammonium oxalate 2.5 g L^{-1}); pH (1 and 3.5); S/L (solid to liquid ratio 1:4, 1:8, 1:12). The extraction time was 18 h in all the trials.

[c] 2-*O*-Me Fuc: 2-*O*-Me-Fucose; 2-*O*-Me Xyl: 2-*O*-Me-Xylose; GalA: Galacturonic acid; GlcA: Glucuronic acid.

[d] Total carbohydrates as the sum of individual monosaccharides.

Table 3
Polysaccharide concentration (mg g^{-1} of grape extract)[a] of twelve grape extracts determined by GC-MS and extraction yield of the polysaccharide extraction.

Solvent[b]	pH[b]	S/L[b]	PRAG[c]	RG-II[c]	HG[c]	GP[c]	TP[c]	PEE[d]
TA	pH 1	1:4	**157.7 (15.61)** γ	69.4 (14.62) β	87.7 (7.51) γ	175.2 (16.50) β	**490.1 (27.97)** γ	46.0
TA	pH 1	1:8	90.6 (6.79) β	74.1 (19.01) β	**44.1 (4.80)** β	145.5 (55.12) β	354.2 (58.92) β	29.7
TA	pH 1	1:12	41.0 (8.02) α	**26.5 (3.44)** α	**18.1 (2.72)** α	**58.7 (29.87)** α	**144.2 (31.33)** α	13.0
TA	pH 3.5	1:4	135.5 (0.93) Σ	**73.64 (6.92)** Σ	**31.1 (3.03)** Δ	171.8 (36.81) Δ	412.0 (37.62) Σ	39.2
TA	pH 3.5	1:8	**89.9 (8.91)** Δ	**62.6 (4.98)** Δ	**18.5 (3.79)** Δ	**148.9 (19.76)** Δ	**319.9 (22.57)** Γ	29.7
TA	pH 3.5	1:12	**37.7 (0.29)** Γ	**26.5 (2.08)** Γ	**17.4 (2.66)** Γ	**19.8 (8.23)** Γ	**101.4 (8.91)** Γ	9.2
		pH (%)	0.96	0.31	30.30	1.10	3.38	
		p-value	0.009	0.506	0.000	0.242	0.002	
		S/L (%)	96.67	89.55	47.92	87.94	93.51	
		p-value	0.000	0.000	0.000	0.000	0.000	
		pH × S/L (%)	1.16	2.33	20.75	2.24	0.45	
		p-value	0.018	0.208	0.000	0.254	0.391	
AO	pH 1	1:4	**226.3 (13.74)** c	77.6 (17.13) a	81.3 (17.96) b	172.9 (16.42) a	**558.15 (32.82)** c	50.8
AO	pH 1	1:8	98.2 (14.87) b	77.1 (3.71) a	**74.5 (21.27)** b	130.3 (54.80) a	380.1 (60.79) b	41.1
AO	pH 1	1:12	34.1 (4.62) a	**82.4 (22.20)** a	**5.0 (4.04)** a	**169.6 (8.99)** a	**291.0 (24.74)** a	23.5
AO	pH 3.5	1:4	143.5 (30.57) B	**128.2 (23.99)** B	**9.6 (7.31)** A	157.4 (9.46) A	438.7 (40.67) B	31.7
AO	pH 3.5	1:8	**70.5 (2.80)** A	**134.2 (25.86)** B	**7.8 (6.19)** A	**203.9 (8.83)** B	**416.4 (28.20)** B	31.6
AO	pH 3.5	1:12	**54.4 (6.32)** A	**76.0 (5.03)** A	**12.0 (2.93)** A	**145.5 (13.10)** A	**287.8 (15.61)** A	23.3
		pH (%)	5.33	38.23	42.64	4.30	2.20	
		p-value	0.000	0.000	0.000	0.197	0.120	
		S/L (%)	82.38	18.91	24.20	2.30	76.86	
		p-value	0.000	0.008	0.000	0.619	0.000	
		pH y S/L (%)	10.44	27.33	28.72	65.79	11.52	
		p-value	0.000	0.002	0.000	0.001	0.008	

[a] Values are means with their standard deviations in parentheses (n = 3). Bold font in the same column indicate statistically significant differences ($p < 0.05$) between the extraction solvent (TA and AO) with the same pH and S/L. Different letters in the same column indicate statistically significant differences ($p < 0.05$). Greek lower-case letters compare separately S/L for pH 1 for TA solvent. Greek upper-case letters compare separately S/L for pH 3.5 for TA solvent. Latin lower-case letters compare separately S/L for pH 1 for AO solvent. Latin upper-case letters compare separately S/L for pH 3.5 for AO solvent. A one-way analysis of variance (ANOVA) was used to compare the effect of the extraction solvent, and a two-way analysis of variance (MANOVA) was used to compare the effect of the pH and the S/L. A Duncan post-hoc test was used to determine significant differences. Italic font indicates the results of the MANOVA analysis and percentage of attributable variance (%).

[b] Solvent (TA: aqueous tartaric acid 2.5 g L^{-1}, AO: aqueous ammonium oxalate 2.5 g L^{-1}); pH (1 and 3.5); S/L (solid to liquid ratio 1:4, 1:8, 1:12). The extraction time was 18 h in all the trials.

[c] PRAG: Polysaccharide rich in arabinose and galactose; RG-II: rhamnogalacturonans type II; HG: homogalacturonans; GP: glucosyl polysaccharides (celluloses and hemicelluloses); TP: total polysaccharides as the sum of PRAG, RG-II, HG, GP and TP.

[d] PEE: Polysaccharide Extraction Efficiency calculated as mg of total carbohydrates (calculated by GC–MS) per g of extract.

results on the extraction of grape polysaccharides as no significant differences were observed between solvents regarding the extraction of total polysaccharides. Therefore, both solvents were initially selected. Acid pH values showed better extraction yields and were also selected (pH 1 and pH 3.5). Higher solid to liquid ratio seemed to be critical for higher extractions with aqueous tartaric acid but for ammonium oxalate, and thus all ratios were studied. Larger extraction times increased the polysaccharide extraction with aqueous ammonium oxalate and were needed for the extraction of smaller polysaccharides; thus, 18 h of extraction time was chosen. As solvent concentration did not show any relation with the extraction of total polysaccharides, the lowest concentration (2.5 g L^{-1}) was used in order to reduce the amount of reagent. All these parameters were selected for the extractions. and the resulting extracts analyzed by GC-MS. The analyses of these samples were carried out in triplicate to determine their glycosyl residue composition (Table 2) and polysaccharide families (Table 3).

Table 2 shows the total carbohydrate content and monosaccharide composition (mg carbohydrates g^{-1} of grape extract) of the extracts obtained with the different conditions selected. In all samples, glucose and galacturonic acid, along with and galactose, were the major carbohydrates, with values in accordance with previous researches (Apolinar-Valiente et al, 2010). The high content of glucose was attributed to grape structural glucosyl polysaccharides (GP) as celluloses and hemicelluloses, which are mainly xyloglucans in grape skins (Doco et al., 2003; Pinelo et al., 2006). The xylose residues detected were thus components of xyloglucans. The content of galacturonic acid was mainly attributed to homogalacturonans (Ayestarán et al., 2004).

Rhamnose, arabinose and glucuronic acid were also present in important amounts and in the ranges described in bibliography (Ortega-Regules et al., 2006; Apolinar-Valiente et al., 2010, 2015), although a comparison is difficult because the content of carbohydrates in the grape pomace is clearly dependent on the variety, the grape ripeness and the method of extraction, and all these studies analyze just grape skins and no grape pomaces. The glycosyl derivates of arabinose, galactose, rhamnose and glucuronic acid were used to estimate the content of PRAG in the samples, considering also the molar ratios of the RG-II (Ayestarán et al., 2004). Minor carbohydrates, 2-*O*-methyl xylose, 2-*O*-methyl fucose, aceric acid, apiose, DHA and Kdo, were also detected in all the extracts, and were used to estimate the content of RG-II as described in bibliography (Ayestarán et al., 2004). The presence of mannose was associated with mannans, which are polysaccharides from the grape pericarp formed by linear chains made up of β-1,4-linked mannose units (Vidal et al., 2001; Arnous & Meyer, 2009). The content of this carbohydrate was in the range described in bibliography for grape skins of different varieties (Apolinar-Valiente et al., 2010, 2015).

The monosaccharide composition of the different extracts showed significant differences depending on the conditions used, which was reflected in the amount of the different polysaccharide families (Table 3). Total polysaccharides (TP) were calculated as the sum of polysaccharides rich in arabinose and galactose (PRAG), rhamnogalacturonan type II (RG-II), homogalacturonans (HG) and glucosyl polysaccharides (GP).

Firstly, a one-way analysis of variance was applied to compare the global effect of the extraction solvent, tartaric acid (TA) and ammonium oxalate (AO). The use of TA or AO solvents did not show significant differences in the extraction of total polysaccharides (p-value = 0.056),

Table 4
Estimated polysaccharide concentration (mg g^{-1}) of twelve extracts determined by HPSEC-RID on a Superdex-75 GL column [a].

			Mean Molecular Mass (kg mol^{-1})				
			55.0	12.7	5.4	3.6	
Solvent[b]	**pH**[b]	**S/L**[b]	PEAK 1	PEAK 2	PEAK 3	PEAK 4	ETP[c]
TA	pH 1	1:4	**123.8 (26.96)** β	**0 (0)**	0 (0)	0 (0)	**123.8 (26.96)** β
TA	pH 1	1:8	**100.94 (19.52)** α	**0 (0)**	0 (0)	0 (0)	**100.9 (19.52)** αβ
TA	pH 1	1:12	**62.56 (4.62)** α	0 (0)	0 (0)	0 (0)	**62.6 (4.62)** α
TA	pH 3.5	1:4	135.91 (9.88) Δ	**0 (0)**	**0 (0)**	**0 (0)**	135.9 (9.88) Δ
TA	pH 3.5	1:8	**132.94 (6.97)** Δ	**0 (0)**	**0 (0)**	**0 (0)**	**132.9 (6.97)** Δ
TA	pH 3.5	1:12	**21.5 (6.92)** Γ	**0 (0)**	**0 (0)**	**0 (0)**	**21.5 (6.92)** Γ
		pH (%)	0.01				0.01
		p-value	0.887				0.889
		S/L (%)	79.56				79.54
		p-value	0.000				0.000
		pH × S/L (%)	12.62				12.64
		p-value	0.003				0.003
AO	pH 1	1:4	**325.0 (25.23)** c	**248.9 (13.07)** c	0 (0)	0 (0)	**573.9 (28.41)** c
AO	pH 1	1:8	**162.0 (12.87)** b	**213.6 (18.92)** b	0 (0)	0 (0)	**375.5 (22.88)** b
AO	pH 1	1:12	**39.45 (3.90)** a	0 (0)a	0 (0)	0 (0)	**39.4 (3.90)** a
AO	pH 3.5	1:4	127.5 (27.72) B	**48.6 (9.62)** C	**0.5 (0.08)** A	**0.7 (0.17)** A	177.3 (29.34) B
AO	pH 3.5	1:8	**64.03 (12.41)** A	**14.05 (5.74)** B	**1.3 (0.42)** B	**0.4 (0.13)** A	**79.78 (13.68)** A
AO	pH 3.5	1:12	**163.8 (15.18)** B	**1.76 (0.56)** A	**0.8 (0.20)** A	**0.6 (0.22)** A	**166.9 (15.19)** B
		pH (%)	9.33	41.08	70.33	81.48	25.73
		p-value	0.000	0.000	0.000	0.000	0.000
		S/L (%)	36.28	37.16	10.20	3.95	36.01
		p-value	0.000	0.000	0.012	0.150	0.000
		pH × S/L (%)	51.88	21.09	10.20	3.95	37.42
		p-value	0.000	0.000	0.012	0.150	0.000

[a] Values are means with their standard deviations in parentheses (n = 3). Bold font in the same column indicates statistically significant differences ($p < 0.05$) between the extraction solvent (TA and AO) with the same pH and S/L. Different letters in the same column indicate statistically significant differences ($p < 0.05$). Greek lower-case letters compare separately S/L for pH 1 for TA solvent. Greek upper-case letters compare separately S/L for pH 3.5 for TA solvent. Latin lower-case letters compare separately S/L for pH 1 for AO solvent. Latin upper-case letters compare separately S/L for pH 3.5 for AO solvent. A one-way analysis of variance (ANOVA) was used to compare the effect of the extraction solvent, and a two-way analysis of variance (MANOVA) was used to compare the effect of the pH and the S/L. A Duncan post-hoc test was used to determine significant differences. Italic font indicates the results of the MANOVA analysis and percentage of attributable variance (%).

[b] Solvent (TA: aqueous tartaric acid 2.5 g L^{-1}, AO: aqueous ammonium oxalate 2.5 g L^{-1}); pH (1 and 3.5); S/L (solid to liquid ratio 1:4, 1:8, 1:12). The extraction time was 18 h in all the trials.

[c] ETP: estimated total polysaccharides as the sum of polysaccharides of different molecular mass (peak 1 + peak 2 + peak 3 + peak 4).

and PRAG (p-value = 0.520) and GP families (p-value = 0.666). However, and in accordance with what was described in the previous sections, it affected the extraction of smaller polysaccharides as RG-II (p-value = 0.000) and HG (p-value = 0.013).

On the other hand, a one-way analysis was also applied to compare the effect of the extraction solvent among the samples with the same pH and S/L conditions. A multivariate analysis of variance (MANOVA) was conducted in the samples grouped by the extraction solvent to analyze the effect of the pH and the S/L on the monosaccharide and polysaccharide families. A Duncan post-hoc tests was applied to determine significant differences among the samples extracted with different S/L (Tables 2, 3 and 4).

In the MANOVA results grouped by the TA solvent (Table 3), the S/L ratio showed the major contribution to the observed variation for all the polysaccharide families. The contribution of pH was high for HG. Although the effect of the pH represented a small fraction of the variation in PRAG and TP, the p-values confirmed that this variable was statistically significant. On the other hand, the MANOVA results grouped by the AO solvent showed that the pH effect presented the major frequencies for RG-II and HG, while in PRAG the major effect was attributed to the S/L ratio.

The highest concentrations of TP were obtained with both solvents at pH values of 1 and with 1:4 solid to liquid ratios, and were higher than those described in other studies with different methods of extraction (Apolinar-Valiente et al., 2010).

Regarding to the PRAG content, the highest concentrations were obtained with these conditions, and the same occurred for homogalacturonans. For the RG-II molecules, similar values were obtained in all the extracts except for ammonium oxalate pH 3.5 and 1:4 and 1:8 S/L ratios, which showed the highest values; and tartaric acid 1:12 S/L ratios, which showed the lowest amounts. The extracts obtained with tartaric acid and 1:12 S/L ratios also showed the lowest concentrations of glucosyl polysaccharides; the rest of the trials did not show significant differences in the content of these polysaccharides, except for the GP obtained with ammonium oxalate pH 3.5 and 1:8 S/L, which presented the highest value in these polysaccharides.

Table 4 shows the polysaccharide concentration (mg g^{-1}) estimated by HPSEC-RID in the twelve extracts selected. A Pearson correlation analysis was applied to correlate these data with those obtained by the GC-MS (Table 3). Positive correlations were found between total polysaccharides calculated by GC-MS (TP) and total polysaccharides estimated by HPSEC (ETP) (Pearson correlation coefficient of 0.738, p-value = 0.006). Moreover, positive correlations were also found between PRAG values obtained by GC-MS and polysaccharides of peak 1 estimated by HPSEC (Pearson correlation coefficient of 0.818, p-value = 0.001), which was expected as PRAG is the major polysaccharide of peak 1. Peak 2 also showed a positive correlation with the content of RG-II (Pearson correlation coefficient of 0.690, p-value = 0.013), also expected as this polysaccharide elutes in this peak. On the contrary, no correlations were found for peaks 3 or 4 because these peaks are a mixture of all polysaccharide families and their fragments (see *Section 3.1*).

3.4. Method efficiency

Two parameters were calculated to analyze the method efficiency. The *extraction efficiency* was calculated as the amount of the extract obtained from the white pomace (mg of extract per g of grape pomace). The *polysaccharide extraction efficiency* was calculated as the amount of total polysaccharides obtained from the extracts (mg of total

polysaccharides calculated by GC-MS per g of extract).

The average extraction efficiency of the trials yielded 65.3 ± 13.1 mg extract per g of grape pomace, which is slightly lower than those obtained in some methodologies developed for analytical purposes (Apolinar-Valiente et al., 2010). However, it has to be remarked that these methods use grape skins and not grape pomace, which also contains rest of pulp, seeds and even stems. The polysaccharide extraction efficiency (PEE) of the trials (Table 3) yielded similar values or even higher than those obtained in other analytical methods (Apolinar-Valiente et al., 2010). The highest efficiencies were obtained with both solvents at pH 1 and 1:4 solid to liquid ratio (Table 3). With these conditions, both extractions showed higher values of polysaccharide extraction efficiency than those described in bibliography (Apolinar-Valiente et al., 2010) and allowed the extraction of all the polysaccharide families (PRAG, RG-II, HG, GP) in quite similar proportions.

Considering all the results, the optimum conditions were TA as solvent of extraction, 2.5 g L^{-1} solvent concentration, pH = 1, 1:4 solid to liquid ratio, and 18 h of extraction time, as they allowed to use food safe procedures and yielded to high efficiencies. These conditions supposed a use of 0.15 g of chelating agent per g of grape pomace, 0.60 mL of water per g of grape pomace, and 4 mL of acidic ethanol per g of grape pomace.

4. Conclusions

This paper aims to optimize a method to extract polysaccharides from white grape pomace (non-fermented), analyzing different factors that could affect their extraction. Both tartaric acid (TA) and ammonium oxalate (AO) showed similar effectiveness for the extraction of total polysaccharides, although AO enhanced the extraction of smaller polysaccharides when high pH values were applied. Acid pH values increased the extraction of total polysaccharides and high (average *Mw* = 55 kg mol^{-1}) and medium molecular weight polysaccharides (average *Mw* = 12.7 kg mol^{-1}), and neutral pH produced a higher extraction of smaller fragments (≤5.4 kg mol^{-1}). Longer extraction times enhanced the extraction of polysaccharides for AO extracts, and were needed to release the smaller polysaccharides. The highest efficiencies were obtained with both solvents at pH 1 and 1:4 solid to liquid ratio. The optimum conditions selected (TA as solvent of extraction, 2.5 g L^{-1} solvent concentration, pH = 1, 1:4 solid to liquid ratio, and 18 h of extraction time) allowed to use food safe procedures and reduce solvent consumption.

This paper describes for the first time a method to recover grape polysaccharides from grape pomace, the main by-product of the wine industry. It allows the extraction of all grape polysaccharides (PRAG, RG-II, HG, GP and GL) under efficient and food-safe conditions, which is of interest for the industrial winemaking waste management. Future studies are needed to know the effect of the extracted compounds on the organoleptic and chemical properties of the wines, and to know their enological use.

CRediT authorship contribution statement

Diego Canalejo: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **Zenaida Guadalupe:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing. **Leticia Martínez-Lapuente:** Data curation, Formal analysis, Methodology. **Belén Ayestarán:** Conceptualization, Investigation, Methodology, Supervision, Writing - review & editing. **Silvia Pérez-Magariño:** Conceptualization, Funding acquisition.

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.

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