

Re-investigation of the relevant factors affecting quantitative precipitation of wine polysaccharides: A GC–MS and HRSEC–RI characterization study using central composite design and response surface methodology[☆]

Riccardo Savastano^{a,b,c,*}, Nathalie Vivas de Gaulejac^d, Magali Picard^d, Nicola Francesca^b, Eric Fouquet^a, Nicolas Vivas^a

^a Université de Bordeaux, CNRS - UMR 5255, Institut des Sciences Moléculaires, 351 Cours de la Libération, 33405 Talence Cedex, France

^b Department of Agricultural, Food and Forest Sciences (SAAF), University of Palermo, Viale delle Scienze, Bldg. 5, Palermo 90128, Italy

^c HTS enologia, Contrada Amabilina, 218 A, Marsala 91025, Italy

^d Centre de Recherche Demptos, ISM-CESAMO, Université de Bordeaux, 351 cours de la Libération, 33405 Talence, France

ARTICLE INFO

Keywords:

Polysaccharides
Wine
Precipitation
Central composite design
Response surface methodology

ABSTRACT

Wine polysaccharides are abundant and complex macromolecules that influence wine's colloidal stability and quality. Although several precipitation methods have been proposed for their quantification, optimal conditions for quantitative recovery have not yet been systematically evaluated using central composite design and response surface methodology. In this study, six constitutional monosaccharides were quantified by gas chromatography–mass spectrometry to assess the effects of sample concentration, precipitation time, precipitation agent composition and their interactions on wine polysaccharide recovery, while high-resolution size-exclusion chromatography with refractive index detection complemented this assessment by providing polymer distribution data. All quadratic models were highly significant ($p < 0.0001$) and showed good predictive performance. Precipitation time had no significant effect, whereas moderate sample concentration coupled with mildly acidified ethanol enhanced the recovery and analysis of rhamnogalacturonans and polysaccharides rich in arabinose and galactose. Mannoproteins precipitated efficiently even in highly acidified non-concentrated samples, whereas galacturonic acid exhibited an opposite trend, indicating that homogalacturonans may require specific and less common conditions for full recovery. This work allowed a partial optimisation, highlighting the potential need for multi-step protocols to recover the full spectrum of wine polysaccharides.

1. Introduction

Polysaccharides are one of the major classes of macromolecules present in wines, alongside tannins and proteins (Jones-Moore et al., 2022; Zhai et al., 2023). Their concentration typically ranges between 0 and 2 g/L, depending on several factors such as vintage, grape variety, climate and winemaking techniques employed during vinification (Apolinar-Valiente et al., 2014; González-Royo et al., 2013; Guadalupe & Ayestarán, 2007; Jones-Moore et al., 2021). With complex and diverse structures, wine polysaccharides are generally categorised as either grape- or yeast-derived. They exhibit molecular sizes ranging from 5 to 800 kDa and can be composed of up to 15 different monosaccharides

(Doco et al., 2001). Grape-derived polysaccharides are solubilised in must from the complex pectocellulosic cell wall material of grape skins and pulp, following enzymatic depolymerisation occurring during grape maturation and the early stages of vinification (Doco et al., 1999; Doco et al., 2001; Jones-Moore et al., 2021; Zhai et al., 2023). Although grape berries contain a large number of polysaccharides, many are enzymatically degraded and/or precipitated during maceration and alcoholic fermentation (Guadalupe & Ayestarán, 2007). Thus, the most abundant grape-derived polysaccharides found in red wine are arabinogalactan proteins and rhamnogalacturonans (RG-II and RG-I) (Ayestarán et al., 2004; Vidal et al., 2003). Other grape polysaccharides that might be found in wine include arabinans, arabinogalactans (AG-I and AG-II),

[☆] This article is part of a Special issue entitled: '20th ICPG Conference' published in Carbohydrate Polymers.

* Corresponding author at: Université de Bordeaux, CNRS - UMR 5255, Institut des Sciences Moléculaires, 351 Cours de la Libération, 33405 Talence Cedex, France.

E-mail address: riccardo.savastano@u-bordeaux.fr (R. Savastano).

<https://doi.org/10.1016/j.carbpol.2025.124326>

Received 19 June 2025; Received in revised form 18 August 2025; Accepted 29 August 2025

Available online 30 August 2025

0144-8617/© 2025 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

galactans, homogalacturonans (HG), and hemicellulosic polysaccharides, such as xyloglucans (Doco et al., 1999, 2003; Jones-Moore et al., 2021; Vidal et al., 2003). On the other hand, yeast-derived polysaccharides are mainly represented by mannoproteins (MPs) which are released in wine along with mannans and glucans as a consequence of yeast autolysis or due to the addition of yeast derivatives during alcoholic fermentation and wine aging (Escot et al., 2001; González-Royo et al., 2013; Guadalupe et al., 2012). Additionally, glucans produced by *Botrytis cinerea* can also be present if infected grapes are vinified as well as arabic gum and carboxymethylcellulose (CMC) coming from exogenous addition of authorised additives (Guadalupe et al., 2012). These macromolecules have been isolated, characterized and studied in some depth over the past 30 years due to their key role in modulating wine's colloidal state, stability and overall quality (Jones-Moore et al., 2022; Zhai et al., 2023). Indeed, wine polysaccharides are known to interact with several wine constituents such as polyphenols (Brandão et al., 2017; Guadalupe & Ayestarán, 2008; Li et al., 2019; Manjón et al., 2021; Riou et al., 2002; Rodrigues et al., 2012), proteins (Dupin et al., 2000; Moine-Ledoux & Dubourdieu, 1999; Waters et al., 1994a; Waters et al., 1994b), tartaric acid salts (Gerbaud et al., 1996) and aroma compounds (Canalejo et al., 2024; Dufour & Bayonove, 1999; Lubbers et al., 1994; Mitropoulou et al., 2011). The isolation of these macromolecules from the wine matrix is typically achieved through precipitation induced by ethanolic dehydration and represents a critical preliminary step in both their quantification and characterization (Zhai et al., 2023). In quantitative analyses, total soluble polysaccharides must be precipitated using a relatively simple, yet robust and reproducible method that ensures an accurate representation of wine's polysaccharidic fraction while allowing the analysis of multiple samples in series. Quantitative precipitation methods have thus evolved over the past decades to meet these requirements, with several protocols proposed for this purpose. These methods primarily differ in three aspects related to sample preparation and processing including the concentration rate of wine achieved prior to precipitation, the duration of the precipitation process and the composition of the precipitation agent (Ayestarán et al., 2004; Doco et al., 1999; Guadalupe et al., 2012). In the present study, we re-investigated the relevance of these parameters, as well as their interactions, using a central composite design (CCD). We hypothesized that variations in these parameters would significantly affect wine colloid precipitation, resulting in precipitates with distinct compositions, which would be reflected in polysaccharide quantification. Therefore, the products of multiple precipitations were systematically characterized for both monosaccharide composition and polymer size distribution. To this end, gas chromatography–mass spectrometry (GC–MS) and high-resolution size-exclusion chromatography coupled with refractive index detection (HRSEC–RI) were employed. Response surface methodologies (RSM) were then applied to explore the experimental domain, with the goal of identifying conditions that maximize extraction efficiency and analytical response.

2. Materials and methods

2.1. Standards and reagents

All standards and reagents were analytical-reagent grade unless otherwise stated. Standards of different monosaccharides D-mannose, D-glucose, D-glucuronic acid, D-galacturonic acid, D-galactose, D-xylose, L-fucose, L-arabinose, L-rhamnose and myo-inositol were supplied by Sigma-Aldrich (Saint-Quentin Fallavier, France). HPLC-grade methanol used for preparing methanolic HCl and HPLC-grade hexane were purchased from Thermo Fisher Scientific (Villebon-sur-Yvette, France) while calcium hydride, acetyl chloride and hydrochloric acid 37 % from Sigma-Aldrich (Saint-Quentin Fallavier, France). Ethanol 96 % v/v was supplied from VWR Chemicals (Fontenay-sous-Bois, France), Tri-Sil™ HTP Reagent was bought from Thermo Fisher Scientific (Villebon-sur-Yvette, France) and lithium nitrate HPLC-grade from Sigma-Aldrich

(Saint-Quentin Fallavier, France). Shodex P-82 kit composed of six pullulan calibration standards (P-5, Mw = 6.4 kDa, P-10, Mw = 9.8 kDa; P-20, Mw = 21.9 kDa; P-50, Mw = 49.4 kDa; P-100, Mw = 107 kDa; P-200, Mw = 218 kDa) was obtained from Resonac (Tokyo, Japan), while pullulan with nominal Mw = 1.26 kDa (P-1) was obtained from Agilent Technologies (Santa Clara, CA, USA).

2.2. Design of experiments

The experimental design was set up with the aim to optimize the quantitative precipitation protocol for the extraction of total soluble wine polysaccharides. Sample concentration rate (A), precipitation time (B) and the concentration of HCl in ethanol used to induce the precipitation (C), were the three factors considered in a second order symmetrical design. A CCD was utilised, with 3 levels for each factor coded as -1, 0, 1 (Table 1). The design was composed of 18 experimental runs, conducted in a random order, consisting in 8 factorial, 6 axial combinations and 4 repetitions of the central point. Two additional experimental runs, for which levels were set based on optimum condition present in literature (Ayestarán et al., 2004), 19 (A = 0.8; B = 0; C = 1) and 20 (A = 0.8; B = -1; C = 1) were also realised in order to test the prediction ability of the models. The concentration (mg/L) of several monosaccharides present in wine polysaccharides including rhamnose, 2-O-methyl fucose, galacturonic acid, arabinose, galactose and mannose; were considered as responses.

2.3. Precipitation of wine polysaccharides

Wine polysaccharides were recovered by precipitation after ethanolic dehydration following the different precipitation protocols planned in the experimental design. A Merlot based red wine vinified in Saint-Emilion (France) during 2019 vintage was sampled from an 80 hL stainless steel vat. The wine was clarified by centrifugation (4400 rpm; 20 min; 20 °C) and 10 mL of supernatant were used for each experimental run. Three levels of concentration were achieved as follows: non-concentrated samples (A = -1) were directly precipitated, while concentrated samples at 50 % (A = 0) and 100 % (A = 1) were firstly concentrated to dryness using a rotary evaporator at 35 °C and reduced pressure. The residues from the former were re-dissolved in 5 mL of MilliQ water prior to precipitation, while the residues from the latter were treated with the precipitation agent without any addition of water. Precipitation was induced as previously described (Ayestarán et al., 2004; Doco et al., 1999; Guadalupe et al., 2012) with the addition of four volumes (40 mL) of cold (4 °C) ethanol prepared with three molar concentrations of HCl (Table 1) and occurred at 4 °C for the timings defined by each experimental run. Thereafter, samples were centrifuged (4400 rpm; 20 min; 20 °C), the supernatant was discarded, and the sediment was washed two times with 96 % v/v ethanol at 4 °C. The excess of ethanol was removed, sediments were further dried with a stream of nitrogen, dissolved in 1 mL of MilliQ water and lyophilized. The obtained polysaccharides extracts (PEs) were weighted and stock over P₂O₅ under vacuum until utilisation.

2.4. Identification and quantification of monosaccharides by GC–MS

The monosaccharide composition of each PE was determined by GC–MS of their trimethylsilyl-ester O-methyl glycosyl derivatives

Table 1
Coded and real values for each factor of the CCD.

Independent variables		Factor level		
		-1	0	1
A	Concentration rate (%)	0	50	100
B	Precipitation time (h)	5	14.5	24
C	HCl concentration in ethanol (mM)	0	150	300

obtained after acidic methanolysis and derivatization as previously described (Doco et al., 2001), with some modifications. Anhydrous methanol containing HCl 0.5 M was prepared by distilling HPLC-grade methanol on CaH₂ under argon atmosphere followed by the addition of 35 µL/mL of acetyl chloride. PEs (1 mg) were placed in a thermal resistant glass test tube together with 1.5 mL of methanolysis reagent. All operation were carried out under argon. Tubes were sealed with a Teflon-lined screwcap and the methanolysis reaction was conducted in an oil bath at 80 °C for 18 h under constant agitation. Samples were cooled at room temperature and the excess of reagent was removed using a stream of nitrogen gas. Methyl-glycosides were converted to their corresponding trimethylsilylated (TMS) methyl derivatives by adding an excess (0.3 mL) of Tri-Sil® HTP Reagent which was kept at 80 °C for 20 min under agitation. Once cooled, the reagent was removed with a stream of nitrogen. Residues were extracted with 0.5 mL of hexane and 15 µL of a solution of derivatised myo-inositol was added as internal standard. Samples were then centrifuged to separate insoluble salts formed during derivatisation and 1 µL of the obtained solution was used for the GC–MS analysis. All injections were performed in triplicate. The same protocol was applied to monosaccharide standards with the aim of realising patterns for identification and calibration curves.

GC–MS was performed with a Trace GC Ultra (Thermo Fisher Scientific, Villebon-sur-Yvette, France) gas chromatograph coupled with a ISQ mass spectrometer equipped with a Triplus autosampler (Thermo Fisher Scientific, Villebon-sur-Yvette, France). The chromatographic column was an Optima 5MS (30 m × 0.25 mm × 0.25 µm) of phase 5 % diphenyl – 95 % dimethylpolysiloxane (Macherey-Nagel, Düren, Germany). The oven program started at an initial temperature of 120 °C which was increased at a rate of 50 °C min^{−1} to 170 °C, to 180 °C at a rate of 2 °C min^{−1}, to 190 °C at a rate of 1.3 °C min^{−1} and to 195 °C at 1 °C min^{−1}. Temperature was then raised to 210 °C at 10 °C min^{−1} and finally to 320 °C at 50 °C min^{−1}. The GC injectors were maintained at 230 °C with a 1:20 split ratio. The carrier gas was helium (≥ 99.9999 %) at a flow rate of 1.2 mL min^{−1}. Ionisation was performed by electron impact (EI) mode at 70 eV. The temperatures used were 150 °C for the MS Quad, 200 °C for the MS Source, and 250 °C for the transfer line. Quantification was carried out in selected ion monitoring (SIM) mode. D-mannose, D-galactose and L-rhamnose were quantified with *m/z* 204 ion while L-arabinose and D-galacturonic acid with *m/z* 217 ion. For those monosaccharides for which a commercial analytical standard was not available, identification was done based on mass spectra reported by Doco et al. (2001). Quantification of 2-O-methyl fucose was performed with *m/z* 146 ion using L-fucose calibration curve.

2.5. Analysis of polysaccharides by HRSEC–RI

HRSEC was performed as previously described (Guadalupe et al., 2012) with a Dionex UltiMate 3000 liquid chromatograph (Thermo Fisher Scientific, Villebon-sur-Yvette, France) integrated with a RS autosampler, a Vanquish refractive index (RI) detector and a Dionex UltiMate 3000 Diode Array (UV) detector set at 280 nm (Thermo Fisher Scientific, Villebon-sur-Yvette, France). The UV detector was used qualitatively to distinguish polysaccharides from proteins and polyphenols. Two Shodex OHpack columns, SB-805 HQ and SB-803 HQ (0.8 × 30 cm, Resonac, Tokyo Japan) were connected in series and equipped with a SB-G 6B pre-column (Resonac, Tokyo Japan). A solution of LiNO₃ 0.1 M was used as eluant at 1 mL min^{−1}. PEs were dissolved at a concentration of 0.1 % w/v in LiNO₃ 0.1 M and left overnight at 4 °C to favour polymers rehydration. The day after, the solutions were filtered through a 0.45 µm PTFE filter and 100 µL were used for the analysis.

2.6. Data analysis

The concentrations (mg/L) of the monosaccharides considered as responses were modelled to the independent variables by a quadratic function according to the general equation:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{1 \leq i < j \leq k} \beta_{ij} X_i X_j$$

where *Y* is the measured response variable, *k* is the number of factors, β_0 is the constant term, β_i , β_{ii} and β_{ij} are, respectively, the linear, quadratic and interaction coefficients, *X_i* and *X_j* represent the factors (Bezerra et al., 2008).

The computation allowed to verify the significance of each model term through analysis of variance (ANOVA) and Fisher distribution (F test) with *p* < 0.05. Other model performance parameters such as the coefficient of determination of the model (*R*-squared), the adjusted coefficient of determination (Adj *R*-squared), the coefficient of determination in prediction (Pred *R*-squared) and the adequate precision value (Adeq precision, i.e. an estimation of whether the signal/noise ratio is adequate to explore the design space, with values greater than 4 being desirable) were also calculated. All statistical evaluation were performed with RStudio (version 2024.09.0 Build 375).

3. Results

3.1. Yield and appearance of the polysaccharide extracts

The experimental design investigated how variations in sample preparation and precipitation conditions affect the recovery and analysis of wine polysaccharides.

After wine centrifugation, precipitation protocols resulting from combinations of the selected factors were applied to recover wine polysaccharides. The different experimental conditions led to the recovery of varying amounts of PEs, which also differed in colour and morphology. The highest yields were obtained under conditions 2 and 4, where a 100 % concentration rate was used, and precipitation was carried out with non-acidified ethanol. These conditions yielded 101 and 109 mg, respectively, of a deep purple powder characterized by a high presence of crystals. Conversely, in conditions 6, 8 and 10, which also involved a 100 % concentration rate but used ethanol containing 150 or 300 mM HCl as precipitation agent, lower yields of PEs were obtained (36, 34 and 39 mg, respectively). These extracts also showed a high presence of crystals but were distinguished by a shinier magenta colour. Design points 1, 3 and 13, which shared the same precipitation agent composition as conditions 2 and 4 but involved a lower concentration rate (0 %, 0 % and 50 %, respectively), allowed for the recovery of 41, 40 and 53 mg of PEs. These extracts were similar in colour to those from conditions 2 and 4 but contained fewer crystals and displayed a more flocculent appearance. In conditions 11, 12, 15, 16, 17 and 18, where wine samples were concentrated to 50 % and precipitated using ethanol containing 150 mM HCl for different precipitation times, 15, 15, 17, 16, 15 and 16 mg of PEs were recovered, respectively. Despite the higher HCl concentration, condition 14 yielded a similar amount of PE (14 mg). All these extracts were characterized by a light pink colour and a flocculent, adhesive morphology. Finally, the lowest yields were observed in conditions 5, 7 and 9, which employed acidified ethanol but did not include a prior concentration step. These conditions resulted in 8, 7 and 9 mg of PEs, respectively, all exhibiting a light pink colour and a flocculent and adhesive morphology.

3.2. Composition of the polysaccharide extracts by GC–MS

Monosaccharide composition analysis was carried out by GC–MS using 1 mg of each PE, after acidic methanolysis and TMS derivatisation. The analysis revealed varying signal/noise ratios for all monosaccharides, reflecting the differences in purity of the extracts. Not surprisingly, tartaric acid, found at the beginning of the GC–MS chromatogram as its dimethyl tartarate-2TMS derivative, was a major contaminant in the PEs. The highest signal/noise ratios for the tartaric acid derivative were observed in the samples obtained under conditions

that yielded the greatest amounts of PEs, such as design points 2, 4, as well as 1, 3, 6, 8, 10 and 13. These conditions either involved a 100 % concentration step prior to precipitation or employed non-acidified ethanol as precipitation solvent. Much lower signal/noise ratios for this compound were observed in the remaining samples, particularly in the PEs obtained from conditions 5, 7, 9 and 14, where the tartaric acid derivative was almost undetectable.

Despite the occasionally high presence of contaminants, major structural monosaccharides, such as mannose, arabinose, galactose, rhamnose and galacturonic acid, were consistently detectable within their respective quantification limits across all samples. The presence of rare monosaccharides that serve as markers for RG-II, including aceric acid, 2-O-methyl xylose, 2-O-methyl fucose, apiose, 3-deoxy-D-lyxohexulosaric acid (Dha), and 3-deoxy-D-manno-octulosonic acid (Kdo), was also assessed in all samples. However, only 2-O-methyl fucose was detected within its quantification limit in every condition.

Minor monosaccharides such as xylose and glucuronic acid were detectable in all samples, although their quantification was not always possible. In fact, the EI mass spectra of the TMS methyl derivative of xylose found in the PEs chromatograms matched the one obtained from the injection of pure D-xylose, after acidic methanolysis and TMS derivatisation, only in samples obtained with conditions 2 and 4. In all other cases, an unusually high intensity of the m/z 191 ion was observed, suggesting co-elution of this compound with another unidentified TMS glycosyl derivative. Similarly, the main peak of the TMS methyl derivative of glucuronic acid was found to co-elute with TMS derivatives originated from galactose and mannose, compromising both the quantification accuracy and reliability of this uronic acid. Attempts to improve chromatographic separation did not lead to the solution of this flaw. Therefore, an improvement of the methanolysis protocol may be considered in order to reduce the formation of interfering TMS glycosyl derivatives and permit reliable quantification of these two monosaccharides.

3.3. Monosaccharides quantification and CCD models

The concentration (mg/L) of six constitutional monosaccharides, representative of different classes of wine polysaccharides such as mannoproteins (MPs), polysaccharides rich in arabinose and galactose (PRAGs), rhamnolacturonans (RGs) and homolacturonans (HGAs), were considered as responses. The precipitation protocols tested led to differences in the quantification of the six monosaccharides, as summarized in Table 2.

All responses to the independent variables were modelled using a quadratic function, which included a linear and a quadratic coefficient

for each variable and each pair of variables. Results of the ANOVA analysis of the fitted quadratic models are shown in Table 3.

Only the significant terms that remained after backward elimination are reported. These were retained based on their p -value, while respecting the hierarchy between variables and considering the values of the adjusted coefficient of determination (Adj R -squared). None of the terms associated with the precipitation time had a significant effect and were thus omitted from the final models. However, the elimination of these terms led to a significant lack of fit in all models. To address this flaw an outlier analysis was performed using Cook's distance, which identifies specific design points as influential observations. This approach led to the removal of design point 13 from all models except for the one describing galacturonic acid concentration, where design point 9 was instead excluded. Additionally, for the mannose concentration model, design points 10 and 13 were removed. Models were recalculated after outlier assessment with the reported representing the best obtainable. Each model exhibited high statistical significance with strong R -squared, Adj R -squared, Pred R -squared and Adeq precision values (Table 3). The corresponding quadratic equations are presented in Table 4. Response surfaces describing the concentration of each monosaccharide as a function of concentration rate and HCl content were realised and are shown in Fig. 1.

Two additional points, 19 and 20, for which factors parameters were set based on previously published parameters (Ayestarán et al., 2004), were also incorporated into the experimental design to assess the prediction ability of the models. The factors were set as follows: concentration rate of 80 % for both points; precipitation time of 14.5 h for experimental condition 19 and 5 h for experimental condition 20; and HCl concentration in ethanol of 300 mM for both conditions. The results of the quantification of the six monosaccharides considered as responses for the two additional points were compared with the predicted model values (Table 5). Since no terms related to precipitation time were included in the models, the predicted values were identical for both experimental conditions. Although monosaccharide concentrations were on average slightly lower in condition 20, especially for galacturonic acid, the experimental means for all monosaccharides fell within the prediction intervals (t -test, $P = 95$ %), which confirms the accuracy of the models.

3.4. Polymers distribution analysis by HRSEC-RI

An HRSEC-RI analysis using two Shodex columns connected in series was performed with 1 mg of PEs to obtain further information on the size and distribution of the extracted polysaccharides. Due to high polydispersity, chromatograms exhibited a large number of peaks with

Table 2

CCD matrix and corresponding rhamnose (Rha), 2-O-methyl fucose (meFuc), galacturonic acid (GalA), arabinose (Ara), galactose (Gal) and mannose (Man) values (mg/L).

Design point	Run order	A	B	C	Rha	meFuc	GalA	Ara	Gal	Man
1	12	-1	-1	-1	35.85	1.65	564.14	104.32	59.34	31.04
2	6	1	-1	-1	34.66	1.17	396.15	86.20	60.39	45.75
3	1	-1	1	-1	39.81	1.80	586.86	115.37	62.01	31.94
4	15	1	1	-1	35.51	1.17	412.97	86.78	62.71	48.24
5	2	-1	-1	1	19.82	0.70	78.05	74.55	87.79	42.09
6	13	1	-1	1	19.74	0.75	184.10	50.96	31.79	20.90
7	16	-1	1	1	15.44	0.30	44.71	54.54	72.85	36.92
8	8	1	1	1	16.73	0.62	153.64	44.54	29.24	19.97
9	5	-1	0	0	24.89	1.68	87.32	78.76	84.17	38.57
10	17	1	0	0	18.90	0.69	168.01	50.92	33.81	22.68
11	3	0	-1	0	49.60	2.66	171.03	145.47	98.78	43.72
12	11	0	1	0	44.70	2.33	163.01	136.00	94.57	41.68
13	10	0	0	-1	29.10	1.12	451.25	78.84	50.51	30.33
14	7	0	0	1	45.58	2.48	141.93	122.00	103.16	41.73
15	14	0	0	0	50.20	2.88	181.04	157.41	105.63	45.66
16	4	0	0	0	58.03	3.17	186.22	170.68	108.38	48.75
17	9	0	0	0	45.52	2.60	143.29	133.72	95.54	39.11
18	18	0	0	0	42.28	2.32	149.17	127.03	97.45	40.24

Table 3

ANOVA results for the quadratic models describing the concentration (mg/L) of the six monosaccharides considered as responses as a function of the concentration rate (A), precipitation time (B) and HCl concentration in ethanol (C) together with model performance parameters.

Response	Source	Sum of Squares	df	Mean Square	F value	p-value Prob > F
Rhamnose	Model	2714.09	4	678.52	41.73	<0.0001
	A	10.55	1	10.55	0.65	0.4361
	C	453.15	1	453.15	27.87	0.0002
	A ²	2173.83	1	2173.83	133.70	<0.0001
	C ²	76.55	1	76.55	4.71	0.050
	Residuals	195.11	12	16.26		
	Lack of fit	15.99	3	5.33	0.2678	0.8470
	Pure error	179.12	9	19.90		
	R-squared					0.9329
	Adj R-squared					0.9106
	Pred R-squared					0.8921
	Adeq precision					7.8
2-O-methyl fucose	Model	12.30	4	3.07	37.44	<0.0001
	A	0.30	1	0.30	3.63	0.0809
	C	0.79	1	0.79	9.57	0.0093
	AC	0.27	1	0.27	3.29	0.0949
	A ²	10.94	1	10.94	133.25	<0.0001
	Residuals	0.99	12	0.08		
	Lack of fit	0.35	3	0.12	1.64	0.2481
	Pure error	0.64	9	0.07		
	R-squared					0.9258
	Adj R-squared					0.9011
	Pred R-squared					0.8717
	Adeq precision					7.2
Galacturonic acid	Model	443254.6	4	110813.6	270.2	<0.0001
	A	4678	1	4678	11.40	0.0055
	C	327227	1	327227	797.76	<0.0001
	AC	38761	1	38761	94.50	<0.0001
	C ²	72589	1	72589	176.97	<0.0001
	Residuals	4922	12	410		
	Lack of fit	2036	3	679	2.12	0.1684
	Pure error	2887	9	321		
	R-squared					0.989
	Adj R-squared					0.9854
	Pred R-squared					0.9770
	Adeq precision					24.74
Arabinose	Model	23673.04	3	7891.0	53.47	<0.0001
	A	1169.6	1	1169.6	7.92	0.0146
	C	2462.8	1	2462.8	16.69	0.0013
	A ²	20040.7	1	20040.7	135.79	<0.0001
	Residuals	1918.6	13	147.6		
	Lack of fit	291.7	4	72.9	0.40	0.8018
	Pure error	1626.9	9	180.8		
	R-squared					0.925
	Adj R-squared					0.9077
	Pred R-squared					0.8853
	Adeq precision					8.4
	Model	10821.78	4	2705.4	56.28	<0.0001
Galactose	A	2196.9	1	2196.9	45.70	<0.0001
	C	2.4	1	2.4	0.05	0.8268
	AC	1284.2	1	1284.2	26.72	0.0002
	A ²	7338.3	1	7338.3	152.66	<0.0001
	Residuals	576.8	12	48.1		
	Lack of fit	296.6	3	98.9	3.17	0.0779
	Pure error	280.3	9	31.1		
	R-squared					0.9494
	Adj R-squared					0.9325
	Pred R-squared					0.8986
	Adeq precision					10.41
	Model	1024.56	4	256.14	28.90	<0.0001
Mannose	A	5.77	1	5.77	0.65	0.4368
	C	128.01	1	128.01	14.44	0.0029
	AC	597.78	1	597.78	67.45	<0.0001
	A ²	293.00	1	293.00	33.06	0.0001
	Residuals	97.49	11	8.86		
	Lack of fit	15.3	2	7.65	0.84	0.4638
	Pure error	82.18	9	9.13		
	R-squared					0.9131
	Adj R-squared					0.8815
	Pred R-squared					0.8449
	Adeq precision					8.7

Table 4

Quadratic model equations describing the concentration (mg/L) of rhamnose, 2-O-methyl fucose, galacturonic acid, arabinose, galactose and mannose as a function of the significant factors and their interactions expressed in coded values.

Response	Quadratic model equation
Rhamnose	$48.49 - 1.03 \cdot A - 9.19 \cdot C - 26.9 \cdot A^2 + 5.68 \cdot C^2$
2-O-methyl fucose	$2.69 - 0.17 \cdot A - 0.40 \cdot C + 0.18 \cdot AC - 1.64 \cdot A^2$
Galacturonic acid	$167.98 - 14.10 \cdot A - 180.89 \cdot C + 69.61 \cdot AC + 133.40 \cdot C^2$
Arabinose	$144.79 - 10.81 \cdot A - 21.21 \cdot C - 70.09 \cdot A^2$
Galactose	$100.83 - 14.82 \cdot A - 2.27 \cdot C - 12.67 \cdot AC - 42.42 \cdot A^2$
Mannose	$43.60 - 1.20 \cdot A - 4.33 \cdot C - 8.64 \cdot AC - 8.69 \cdot A^2$

similar retention times, leading to significant coelution. A deconvolution approach was thus evaluated in order to determine peak areas with the aim of modelling these data using a quadratic function, as done for monosaccharide concentration data. Although several deconvolution models were tested, a fitted deconvolution could not be obtained for all chromatograms. An example of a successfully fitted deconvolution using

a Gaussian model is shown in Fig. 2. Nonetheless, this approach permitted the identification of at least 15 distinct peaks in the chromatogram, whose presence and relative abundance varied depending on the experimental condition analysed (Fig. 3).

To facilitate data interpretation, chromatograms were divided into four regions representing distinct molecular weight (Mw) ranges: R1 (t_R 13–17 min), comprising peaks *a*, *b* and *c*; R2 (t_R 17–18.5 min), peaks *d* and *e*; R3 (t_R 18.5–20.5 min), peaks *f* to *i*; and R4 (t_R 20.5–22.8 min), representing the final segment. Integration results for regions R1 to R4 are reported in Table 6. In addition, two indices were calculated to normalize the data and enable comparison across samples: $(R1 + R2)/(R3 + R4)$, reflecting the relative contribution of early- versus late-eluting species; and $R1/R2$, indicating the distribution between high (R1) and medium (R2) Mw polysaccharides (Table 6).

Experimental conditions 7, 9, 11, 12, 14, 15, 16, 17 and 18 showed the highest values for the $(R1 + R2)/(R3 + R4)$ index, indicating a greater relative abundance of high and medium Mw polysaccharides compared to late-eluting species. In contrast, conditions 1, 2, 3, 4, 6, 8, 10 and 13 exhibited the lowest index values, reflecting chromatograms

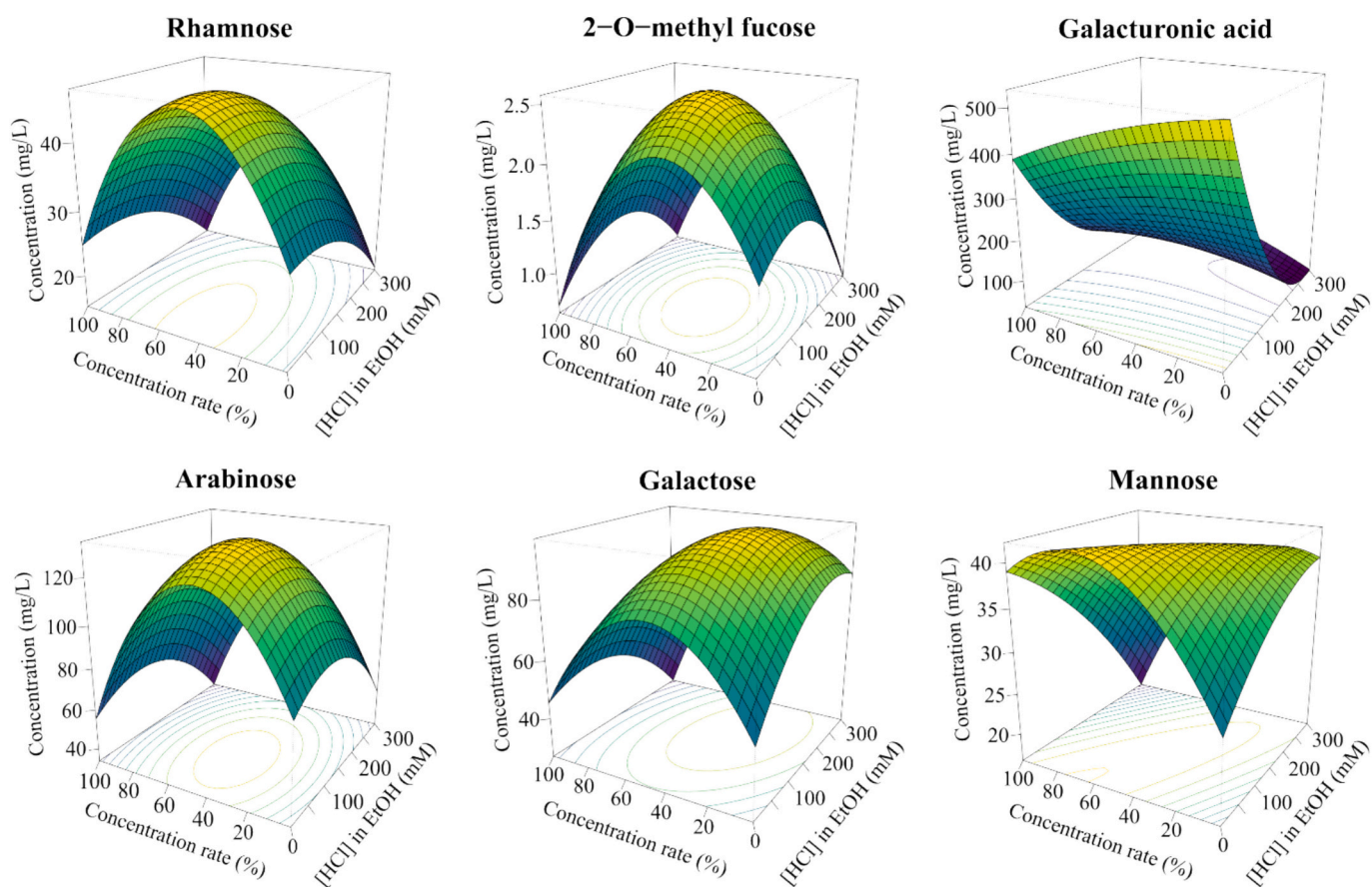


Fig. 1. Response surface plots for rhamnose, 2-O-methyl fucose, galacturonic acid, arabinose, galactose and mannose with varying concentration rate and HCl concentration (precipitation time fixed at 14.5 h).

Table 5

Experimental mean data for the two additional points, 19 and 20, compared to the predicted values.

Response	Exp. 19	Exp. 20	Exp. SD	Lower prediction limit ($P = 95\%$)	Predicted value	Higher prediction limit ($P = 95\%$)
Rhamnose	36.26	32.63	6.84	11.35	26.94	42.53
2-O-methyl fucose	1.76	1.46	0.37	0.38	1.25	2.12
Galacturonic acid	162.12	139.68	21.83	110.60	164.90	219.19
Arabinose	108.89	97.80	20.36	24.35	70.07	115.79
Galactose	62.75	58.29	6.22	33.50	49.41	65.33
Mannose	29.49	26.94	4.55	15.14	25.84	36.54

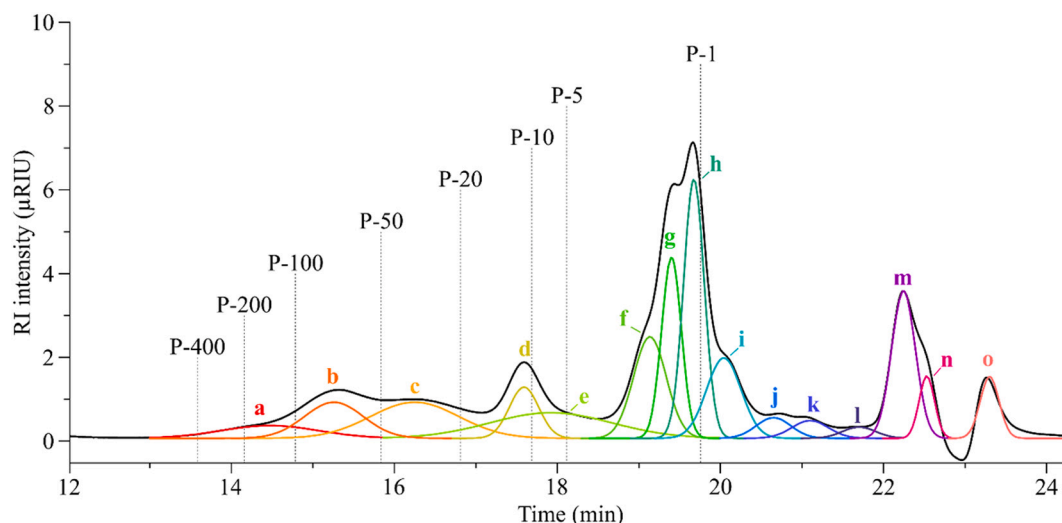


Fig. 2. Example of a successfully fitted HRSEC–RI chromatogram of a wine polysaccharide extract using a Gaussian deconvolution model. Elution times for the pullulan standards are shown (P–400 → P–1).

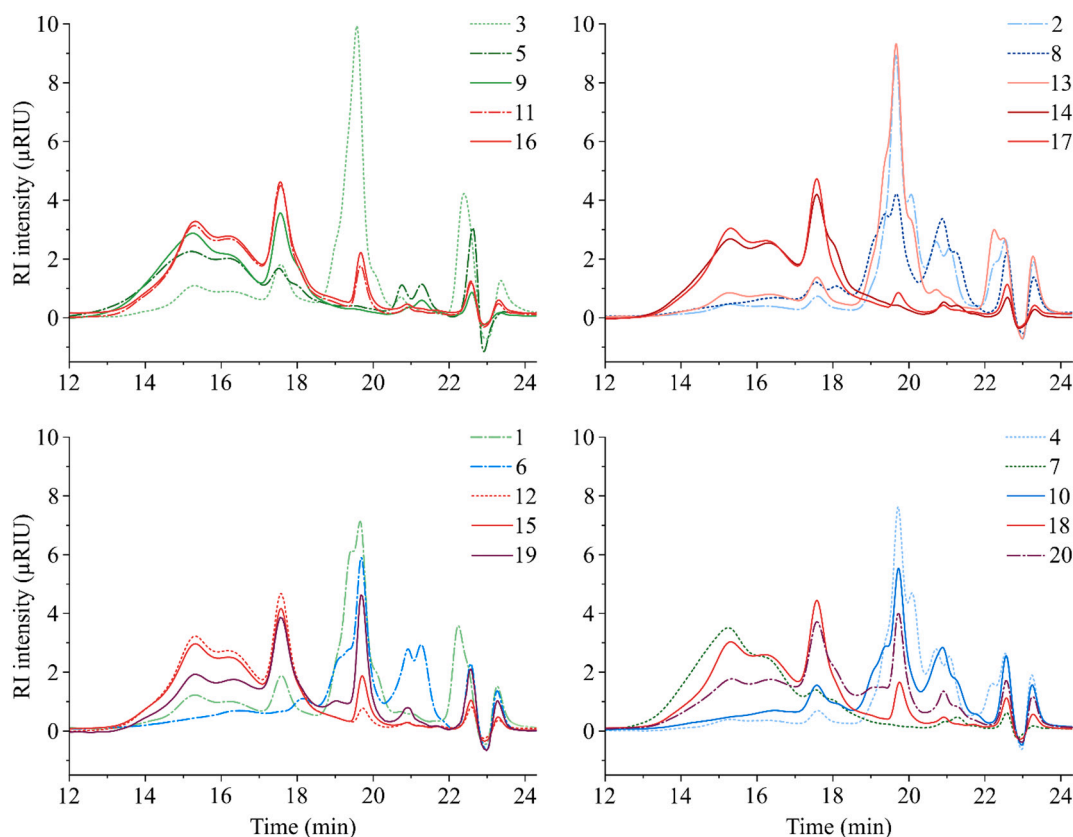


Fig. 3. HRSEC–RI chromatograms of the different polysaccharide extracts. Samples are grouped based on the run order. Concentration rate (A) is represented by colour: green for A = −1, red for A = 0 and blue for A = 1. Precipitation time (B) is indicated by line style: dash-dot for B = −1, solid for B = 0, and dotted for B = 1. HCl content in ethanol (C) is expressed through the intensity of the colour: light for C = −1, medium for C = 0 and dark for C = 1. The two additional experimental conditions (19 and 20), in which A = 0.8, are shown in purple.

predominantly composed of peaks in the R3 and R4 regions. The elution of R3 and R4 peaks near or beyond the column's fractionation limit, combined with HRSEC–UV results showing strong absorbance at 280 nm (data not shown), suggests these regions are not primarily composed of polysaccharides.

Differences among experimental conditions were also observed in the R1/R2 index. The highest R1/R2 values were observed at design

points 5, 7 and 9, indicating a higher relative proportion of high compared to medium Mw polysaccharides. Conversely, conditions 6, 8 and 10 exhibited the lowest values, indicating a greater contribution of the R2 region relative to R1. Additionally, two further groups were identified among the experimental conditions based on R1/R2 values: one comprising conditions 11, 12, 14, 15, 16, 17 and 18, with a relatively high average value of 2.08 ± 0.09 , and another including

Table 6

Integrated HRSEC–RI peak areas for chromatographic regions R1 to R4, along with the two calculated indices.

Design point	R1	R2	R3	R4	(R1 + R2)/(R3 + R4)	R1/R2
1	2.41	1.37	5.84	2.19	0.47	1.76
2	0.92	0.56	5.84	3.51	0.16	1.64
3	2.22	1.34	6.21	2.22	0.42	1.65
4	0.85	0.53	5.59	3.65	0.15	1.61
5	6.34	1.57	0.64	1.78	3.27	4.04
6	1.41	1.08	4.26	3.35	0.33	1.31
7	8.66	1.34	0.44	0.49	10.72	6.46
8	1.39	1.32	4.10	3.53	0.35	1.05
9	7.30	2.88	0.65	0.77	7.16	2.53
10	1.37	1.43	4.21	3.23	0.38	0.95
11	6.97	3.37	1.11	0.51	6.38	2.07
12	7.35	3.41	0.78	0.48	8.55	2.15
13	1.82	1.02	6.59	2.34	0.32	1.79
14	7.07	3.68	0.97	0.59	6.91	1.92
15	6.62	3.04	1.18	0.49	5.81	2.18
16	7.01	3.24	1.35	0.54	5.43	2.17
17	6.99	3.45	1.00	0.68	6.22	2.03
18	6.75	3.26	1.14	0.59	5.79	2.07
19	4.57	3.19	2.70	1.15	2.02	1.44
20	4.29	3.23	2.95	1.41	1.73	1.33

conditions 1, 2, 3 and 4, with a lower average of 1.67 ± 0.07 . Interestingly, HRSEC–UV analysis revealed higher absorbance at 280 nm (data not shown) in the R2 region of experimental conditions with lower R1/R2 values, suggesting that macromolecules other than polysaccharides contribute to the total RI signal in this region when more extreme precipitation protocols are performed. This observation is consistent with the non-selective nature of the RI detector, which does not distinguish analytes based on their chemical nature. A clear example of this limitation is provided by peak *h*, which accounted for a significant portion of the HRSEC–RI signal in samples from conditions 1, 2, 3, 4, 6, 10, 13, 19 and 20. This peak was attributed to tartaric acid, previously found in high abundance in the same samples by GC–MS. Injections of tartaric acid standard solutions confirmed the assignment of peak *h*.

4. Discussion

4.1. Precipitation and isolation of wine polysaccharides

The study of wine polysaccharides requires their prior isolation from the complex wine matrix which is typically achieved through ethanolic precipitation (Ayestarán et al., 2004; Doco et al., 1999; Guadalupe et al., 2012). Although methods for the precipitation of total soluble polysaccharides have been refined over time, a significant need for analytical approaches that combine efficient extraction protocols with accurate qualitative and quantitative measurement still exists (Zhai et al., 2023).

In early characterization studies, polysaccharides were typically isolated from a so called “total colloids” precipitate obtained by addition of cold ethanol to wine (Doco et al., 2001; Pellerin et al., 1996; Vidal et al., 2003). Sample concentration, achieved through ultrafiltration or reduced-pressure evaporation, was employed to recover a larger quantity of material (Vidal et al., 2003), while subsequent purification steps, including dialysis, ion-exchange, size-exclusion and affinity chromatography, were applied to remove salts and to achieve full fractionation of the different polysaccharide families (Doco et al., 2001; Pellerin et al., 1993; Pellerin et al., 1996; Vidal et al., 2003).

Similar methods were later adapted for quantitative analyses, where precision, accuracy, and reproducibility of the precipitation process are essential. In these approaches, different sample concentration rates have been proposed prior to the addition of either ethanol or ethanol acidified with HCl to induce the precipitation (Ayestarán et al., 2004; Doco et al., 1999; Guadalupe et al., 2012). Previous efforts to improve extraction methodologies have primarily relied on one-variable-at-a-time (OVAT) approaches and on indirect quantification methods, such as measuring

the weight of the precipitate, that limit both the understanding of factor interactions and the accurate assessment of polysaccharide content (Ayestarán et al., 2004; Guadalupe et al., 2012). To the best of our knowledge, optimization of wine polysaccharides extraction conditions using a CCD that combines the systematic application of multiple and complementary characterization techniques has not yet been explored.

4.2. Influence of factors on precipitation selectivity and extract nature

The different precipitation protocols resulted in substantial variability in both the yield and visual characteristics of the PEs. These findings strongly suggest that the three variables considered in the CCD influenced the precipitation of distinct wine components, ultimately affecting the composition and purity of the recovered PEs.

As expected, increasing concentration rates led to the recovery of larger amounts of PEs, which is consistent with previous findings (Ayestarán et al., 2004). However, despite the higher yields, the concentration of monosaccharides was systematically lower in conditions where the concentration rate exceeded 50 %, with the exceptions of mannose and galacturonic acid in design points 2 and 4. This indicates that precipitate weight alone is not a reliable indicator of polysaccharide content across all conditions, as the co-precipitation of non-polysaccharidic compounds can significantly affect this measurement. Notably, PEs obtained after full sample concentration (100 %) often contained visible crystalline material, particularly when non-acidified ethanol was employed. This appearance can be attributed to the precipitation of tartaric acid salts, confirmed by both GC–MS and HRSEC–RI analyses, or to the formation of heterogeneous aggregates composed of organic salts, polysaccharides and possibly other wine colloidal constituents such as proteins and polyphenols (Marangon et al., 2025). These compact and heterogeneous aggregates could be less susceptible to acid methanolysis which may explain the reduced yield of methyl glycoside derivatives and the overall lower monosaccharide quantification observed in these samples.

Acidification of ethanol with HCl is a practice absent from most early characterization studies (Doco et al., 2001; Pellerin et al., 1993; Vidal et al., 2003) although it is now widely adopted (Ayestarán et al., 2004; Guadalupe et al., 2012). Despite this, the specific mechanisms by which acidification influences polysaccharides precipitation have not been explicitly addressed in the literature. Our results show that HCl addition improves precipitation selectivity by partially (150 mM) or entirely (300 mM) inhibiting tartaric acid precipitation in non- or partially concentrated (50 %) samples. PEs obtained under acidic precipitation conditions consistently allowed higher monosaccharide quantification by GC–MS when moderate concentration rates (50 %) were reached prior to precipitation. The only exception to this trend was observed for galacturonic acid, whose specific behaviour will be discussed later. Moreover, the HRSEC chromatograms of these conditions were systematically dominated by peaks in the R1 and R2 regions, whereas non-acidified or more highly concentrated conditions showed a predominance of peaks in R3 and R4. The latter regions appear to contain relatively few polysaccharides, as indicated by the strong absorbance at 280 nm, which suggests instead the presence of polyphenols. These compounds are likely to interact only weakly with the SEC stationary phase used, which may explain their late elution near or beyond the columns fractionation limit. In addition, PEs obtained under non-acidified precipitation or when samples were concentrated above 50 %, showed lower R1/R2 ratios, accompanied by elevated 280 nm absorbance in the R2 region, suggesting that the RI signal in this region is not solely due to polysaccharides. It is possible that during precipitation conducted under non-acidified conditions, some free grape-derived proteins (which are known to have a Mw around 20–30 kDa) are also precipitated (Marangon et al., 2025). Unlike polyphenols, these macromolecules are likely to interact with the SEC stationary phase and be effectively fractionated, which could explain the decrease in the R1/R2 index and the higher 280 nm absorbance in R2. Although further

analyses are required to confirm the presence of other wine colloids, such as proteins and polyphenols, in PEs obtained with non-acidified ethanol, their potential presence is consistent with the broader concept of “total colloids”, a term commonly used to describe the products of such precipitation (Doco et al., 2001; Pellerin et al., 1996; Vidal et al., 2003). Overall, moderate concentration (50 %) combined with HCl addition, particularly at 300 mM, appears to enhance precipitation selectivity and analytical response by potentially increasing the solubility of contaminants such as organic acids, polyphenols and proteins, while limiting the formation of compact, heterogeneous aggregates. Such aggregates may be more resistant to acidic methanolysis, potentially leading to an underestimation of the monosaccharide content.

Finally, variation in the precipitation time did not produce any observable effect on precipitation selectivity or the overall composition of the PEs. This suggests that the dehydration and precipitation of wine polysaccharides occur more rapidly than previously assumed (Ayestarán et al., 2004), allowing the precipitation period to be reduced from 18 to 5 h. This reduction shortens an already complex procedure, enhancing practicality without compromising efficiency.

4.3. Optimal precipitation conditions for RGs and HGs

RGs and HGs are grape-derived polysaccharides representing the branched and smooth regions of pectin, respectively (Vidal et al., 2003). RG-II is a major red wine polysaccharide characterized by a (1 → 4)-linked galacturonic acid backbone and highly conserved side chains containing common monosaccharides such as rhamnose, along with rare and diagnostic sugars including 2-O-methyl fucose (Doco et al., 2001). Additional monosaccharides, such as arabinose, galactose and glucuronic acid can also be present in smaller amounts within its side chains (Pellerin et al., 1996). RG-II can occur as a ~ 10 kDa monomer or as a dimer formed via boron-mediated cross-linking through its apiosyl residues (Pellerin et al., 1996). In contrast, RG-I occurs at low concentrations in wines, likely due to incomplete solubilisation or because of its limited resistance to enzymatic degradation (Vidal et al., 2001). Structurally, it consists of a backbone of repeating (1,2)- α -D-galacturonic acid-(1,4)- α -L-rhamnose residues, which can be substituted at the O-4 position of the rhamnose residue with different PRAGs (Jones-Moore et al., 2021; Vidal et al., 2003). In wine, this polysaccharide exhibits an apparent Mw of approximately 40–50 kDa. (Vidal et al., 2003). HGs, on the other hand, are highly abundant in grapes but are rarely detected in finished red or white wines (Vidal et al., 2000). They are composed of linear α -1,4-linked D-galacturonic acid residues that may be partially methyl-esterified or acetylated (Zhai et al., 2023).

Despite the misconception that wine polysaccharides can be strictly divided into neutral and acidic fractions (Doco et al., 1999; Vidal et al., 2000), most galacturonic acid originates from the hydrolysis of RGs and HGs (Pellerin et al., 1996; Vidal et al., 2003), while the majority of rhamnose can be attributed to the presence of RGs (Guadalupe et al., 2012). Although minor amounts of galacturonic acid and rhamnose may derive from other polysaccharide families, such as AGPs (Pellerin et al., 1993; Pellerin et al., 1996), rhamnose was selected to monitor RGs precipitation, 2-O-methyl fucose to specifically track RG-II and galacturonic acid to assess the precipitation of both RGs and HGs.

The models describing rhamnose, 2-O-methyl fucose and galacturonic acid concentrations were highly significant, with satisfactory *R*-squared, adj *R*-squared and pred *R*-squared values. In particular, the galacturonic acid model exhibited the best performance parameter values. Adeq precision values indicated acceptable signal/noise ratios, confirming that the models are reliable for exploring the design space.

Interestingly, the linear term for the concentration rate was not significant in either the rhamnose or 2-O-methyl fucose models, whereas the corresponding quadratic term was highly significant. This indicates that the responses are predominantly influenced by a nonlinear, curvilinear effect of the concentration rate rather than a simple linear trend.

These results align with previous reports showing that RGs and HGs are only partially precipitated in non-concentrated wines (Ayestarán et al., 2004; Doco et al., 1999). Consistently, samples obtained without preliminary concentration yielded lower concentrations of rhamnose and 2-O-methyl fucose, particularly when precipitation was performed with ethanol containing 300 mM HCl. Moreover, these samples exhibited the highest R1/R2 index values, indicating a lack of polysaccharides in R2 (peaks *d* and *e*) with Mw between approximately 10 and 5 kDa, consistent with the reported Mw for RG-II (Vidal et al., 2003). Although five-fold concentration (corresponding to 80 % in the present study) or concentration to dryness (100 %) are often recommended to ensure the quantitative precipitation of RGs and HGs (Ayestarán et al., 2004; Doco et al., 1999; Guadalupe et al., 2012), our results indicate that intermediate concentration rates of 30–60 % are optimal for maximizing the precipitation and quantification of RGs-associated neutral monosaccharides, whereas higher concentration levels (80–100 %) led to underestimation of these sugars. As previously mentioned, this may be due to the formation of heterogeneous aggregates in highly concentrated samples, which could lower the hydrolysis yield during acidic methanolysis. Another possible explanation lies in the higher presence of tartaric acid salts in highly concentrated samples. Inorganic salts are known to interfere with the methanolysis drying step by adsorbing carbohydrate derivatives, thereby reducing quantification accuracy (Chambers & Clamp, 1971; Chaplin, 1982). High levels of organic salts, such as tartrates, may have a similar effect, but the hypothesis warrants further investigation.

Furthermore, no studies have systematically examined the effect of HCl content on polysaccharide precipitation. A first observation from the HRSEC–RI results concerns peak *e*, which displayed improved resolution in highly acidified samples. This might derive from the precipitation of particular polysaccharides under these conditions, although GC–MS data do not show a corresponding increase in monosaccharides. Alternatively, it may suggest that at high HCl concentrations, hydrolysis occurs in higher Mw polysaccharides, potentially affecting the integrity of the RG-II dimer. The hypothesis that RG-II dimers undergo hydrolysis during sample preparation has been previously proposed, but to date it remains difficult to demonstrate (Pellerin et al., 1996; Vidal et al., 2003). Moreover, results from the CCD models show that the linear term for HCl concentration in ethanol was highly significant in both models, whereas the quadratic term was only marginally significant in the model describing rhamnose concentration. Notably, in both rhamnose and 2-O-methyl fucose models, the coefficient for factor C was negative, indicating that RGs are more effectively precipitated when ethanol is non- or only mildly acidified. This behaviour is further supported by the galacturonic acid model, in which the linear term associated with HCl content was highly significant and displayed a strong negative coefficient. Surprisingly, the linear term associated with the concentration rate also exerted a significant negative effect on galacturonic acid quantification. Thus, maximum recovery of galacturonic acid was achieved when samples were not pre-concentrated and precipitation was performed with non-acidified ethanol. Overall, these results suggest that polysaccharides rich in uronic acids (PRUAs) require a non-acidic environment for efficient precipitation. This may be explained by the protonation of galacturonic acid's carboxylic groups in acidic conditions, which increases PRUAs solubility (Vidal et al., 2000). Moreover, the optimal conditions for galacturonic acid quantification differed from those identified for rhamnose and 2-O-methyl fucose, indicating that other PRUAs, beyond RGs, contribute substantially to the total galacturonic acid content. The particular behaviour of galacturonic acid, along with the concurrent presence of lower Mw polymers, suggest the precipitation of HGs fragments. Indeed, conditions yielding the highest galacturonic acid concentrations also produced a characteristic signal in the HRSEC–RI chromatograms (peak *i*). These small HGs polymers may arise from enzymatic hydrolysis of larger pectic structures during winemaking, generating fragments of varying Mw (Pellerin et al., 1996; Zhai et al., 2023). Previous studies have also reported that RG-II can be

linked to other HGs, and that variability in the length of the uronic acid backbone of RG-II may result from incomplete hydrolysis from the HGs to which it is attached (Pellerin et al., 1996). Although further investigation is needed to determine the origin of the increased galacturonic acid under certain conditions, these observations overall suggest that more HGs may be present in wines than previously assumed. However, due to their high solubility in acidic environments, specific and uncommon precipitation conditions are needed for their recovery, which could explain why their presence in wines has often been underestimated.

4.4. Optimal precipitation conditions for PRAGs

PRAGs are another class of pectic, grape-derived polysaccharides that include AGPs, AG-I, AG-II, arabinans and galactans. AGPs, considered the most abundant polysaccharides in red wines, are glycoproteins with variable Mw (50–200 kDa), in which the glucidic component is represented by AG-II, while the protein moiety typically accounts for less than 10 % (Jones-Moore et al., 2021; Pellerin et al., 1993; Vidal et al., 2003). AG-II consists of a (1 → 3)-D-galactan backbone with (1 → 6)-linked side chains, variably substituted by monomeric and oligomeric residues of arabinose and galactose. Other monosaccharides, including rhamnose and galacturonic acid, can be present in AGPs and AG-II side chains in lower concentrations, with galacturonic acid playing a key role in the overall acidity of the macromolecule (Pellerin et al., 1993). In contrast to AG-II, AG-I does not form complexes with proteins to form AGPs and contains only the conventional PRAGs neutral monosaccharides, while arabinans and galactans are homopolymers composed solely of arabinose and galactose, respectively (Jones-Moore et al., 2021). Thus, the concentrations of galactose and arabinose largely reflect the presence of PRAGs (Apolinar-Valiente et al., 2014; Guadalupe et al., 2012) and were used to monitor the precipitation of this polysaccharide group.

No specific precipitation conditions have been reported in the literature to ensure quantitative precipitation of PRAGs. Nevertheless, our CCD models showed that both linear and quadratic terms of the concentration rate significantly affected arabinose and galactose quantification. Optimal precipitation was observed at intermediate concentration rates (30–60 %), whereas both non-concentrated and highly concentrated samples yielded lower levels of these monosaccharides, mirroring the behaviour of RGs-associated neutral sugars. On one hand, the reduced arabinose and galactose concentrations in highly concentrated samples supports the hypothesis of lower acidic methanolysis efficiency under such conditions. On the other hand, the lower concentration of arabinose and galactose in non-concentrated samples suggest that, in this condition, certain PRAGs may also be only partially precipitated, which, to the best of our knowledge, has never been reported before. This specific behaviour may arise from the incomplete precipitation of RG-I in which AG-I, AG-II, arabinans, and galactans can be present as side substitutions (Jones-Moore et al., 2021). Another hypothesis is that lower Mw PRAGs, derived from the hydrolysis of RG-I side chains or larger PRAGs structures, may exhibit greater solubility, requiring higher concentration rates to ensure their precipitation. Moreover, based on the Mw reported for AGPs, these polysaccharides are expected to elute in the R1 region of the HRSEC chromatogram, together with larger MPs (Ayestarán et al., 2004; Guadalupe et al., 2012). Consistently, UV detection revealed a non-negligible absorbance at 280 nm in this region, supporting the presence of protein moieties and lending further credibility to this hypothesis. Interestingly, HRSEC–RI results showed that highly concentrated samples had lower R1/R2 index values compared to moderately concentrated ones. Chromatogram analysis suggests that this difference is more likely due to a depletion of polysaccharides in R1 rather than an enrichment of polymers in R2. This, in turn, may indicate that in highly concentrated samples, larger polymers fail to resolubilise in the solvent used for HRSEC analysis, likely as a result of insoluble aggregate

formation.

Furthermore, the linear term associated with HCl content was significant only in the model describing arabinose concentration, whereas in the galactose model, the AC interaction term was significant. As observed for RGs and HGs, all significant terms associated with factor C had a negative influence. This suggests that arabinose is best quantified when moderate amounts of HCl are added to ethanol, while galactose is efficiently precipitated even at higher HCl concentrations, provided that moderate concentration rates are used.

These observations support the idea that the negative effect of HCl in ethanol is linked to the acidity of the polysaccharide. Less acidic polysaccharides, such as galactans, could be less affected by HCl and precipitate efficiently even at high concentrations. Conversely, PRAGs with higher galacturonic acid content, such as AGPs and AG-II, could be more sensitive to medium acidity and are efficiently precipitated only when lower amounts of HCl are present in the precipitating agent.

4.5. Optimal precipitation conditions for MPs

MPs are the most abundant yeast-derived polysaccharides in wines. They display a wide Mw distribution (5–800 kDa) and are composed primarily of mannose polymers covalently linked to a minor protein backbone via N- or O-glycosidic bonds (Zhai et al., 2023). The mannose released by wine polysaccharide hydrolysis can be fully attributed to MPs (Guadalupe et al., 2012) and was therefore used as a marker of their precipitation.

As with PRAGs, no specific protocol has been reported to ensure complete MPs recovery from the wine matrix. In our models, the mannose response surface revealed a broader range of optimal conditions compared to grape-derived polysaccharides, suggesting that MPs are more consistently precipitated across protocols. The concentration rate showed no significant linear effect, whereas both the quadratic term and the AC interaction were significant. This points to a curvilinear influence, whereby mannose quantification decreases either at high concentration rates combined with high HCl levels, or in non-concentrated samples precipitated with non-acidified ethanol. This behaviour parallels that observed for RGs- and PRAGs-associated neutral monosaccharides.

These findings indicate that precipitation protocols can be tailored to the analytical objective. If the aim is MPs quantification, the concentration step can be omitted, provided that ethanol is strongly acidified. Conversely, when the objective is to simultaneously recover MPs together with major grape-derived polysaccharides, the optimized conditions for RGs and PRAGs can be applied, since MPs also precipitate efficiently under moderate concentration rates coupled with mild acidification.

4.6. Outlier behaviour and repeatability of the methodology

The experimental boundaries of the CCD used in this study were established based on the different precipitation conditions reported in the literature (Ayestarán et al., 2004; Doco et al., 1999; Guadalupe et al., 2012). This approach, however, led to PEs with varying degrees of purity, where co-precipitation phenomena, aggregate formation, and/or the occasional presence of high levels of non-polysaccharidic material could have influenced the efficiency of both the analytical techniques used for their characterization. To achieve non-significant lack of fit values, an outlier analysis was performed for each model, resulting in the removal of one or more design points from the dataset. These outliers are unlikely to reflect analytical errors, as their measured values remain reasonable, but their exclusion was necessary to ensure adequate model performance and predictive ability. This is likely because certain factors and factor interactions were relevant only under specific precipitation conditions, and including the outliers would have increased variability across the models, reducing their overall quality. Nevertheless, the repeatability of the entire methodology, evaluated at the central points,

which corresponded to the optimized conditions for most models, was good, as indicated by standard deviation values, and comparable with previously published data (Guadalupe et al., 2012). The presented models also demonstrated robust predictive performance, further enhancing the reliability of the approach.

5. Conclusions

This study systematically re-evaluated the effects of sample concentration rate, precipitation time, precipitation agent composition and their interactions on the quantitative precipitation of wine polysaccharides. A CCD combined with RSM was employed to model and optimize precipitation conditions, using the concentrations of six constitutional monosaccharides representative of different polysaccharide groups as response variables. Polymer distribution data were also used to evaluate the precipitation and relative abundance of RGs, HGs, PRAGs and MPs in the different PEs obtained.

Our results demonstrate that these parameters not only affect overall precipitate yield but also decisively influence extract purity and analytical response. High concentration rates, although associated with increased yield, promoted co-precipitation of non-polysaccharidic material, reducing monosaccharide quantification by GC–MS. Conversely, moderate concentration rates (30–60 %) combined with ethanol acidification, particularly at 300 mM HCl, enhanced precipitation selectivity by reducing co-precipitation. Precipitation time had no significant effect on polysaccharide recovery, indicating that 5 h suffices to quantitatively precipitate total wine polysaccharides.

Moderate sample concentration rates improved the quantification of rhamnose, 2-*O*-methyl fucose, arabinose and galactose, suggesting better precipitation of RGs and PRAGs and enhanced analytical response during GC–MS and HRSEC–RI analysis. HCl content in ethanol also had a significant effect, with moderate concentrations favouring the precipitation and quantification of RGs and PRAGs. Mannose quantification showed a broader optimal range, indicating efficient MPs precipitation both under conditions optimal for RGs and PRAGs and even in non-concentrated samples when high HCl levels were employed. This highlights the possibility of tailoring precipitation protocols to specific analytical objectives, depending on whether quantification aims to address grape- or yeast-derived polysaccharides, or both.

In contrast, galacturonic acid exhibited an opposite trend, with optimal quantification achieved in non-concentrated samples precipitated with non-acidified ethanol. Our results suggest a higher presence of HGs in wines than previously assumed and indicate that these polysaccharides may require specific precipitation conditions for efficient recovery.

Overall, this study enabled a partial optimisation targeting RGs, PRAGs and MPs, but also indicates that a single-step procedure is unlikely to recover the full polysaccharide spectrum. Optimized multi-step protocols should therefore be evaluated for comprehensive extraction and accurate quantification.

CRedit authorship contribution statement

Riccardo Savastano: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nathalie Vivas de Gaulejac:** Validation, Supervision, Methodology. **Magali Picard:** Software, Data curation. **Nicola Francesca:** Resources, Funding acquisition. **Eric Fouquet:** Supervision. **Nicolas Vivas:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Conceptualization.

Funding

The support for this research was provided through different funding that include: Research Agreement “Biotecnologie Sostenibili per il

Miglioramento della Qualità Tecnologia e Sensoriale dei Vini” between Department of Agricultural, Food and Forestry Sciences e HTS Enologia di Luigi Scavone company; prot.n. 5574 04/10/2021; Project Code CON 0404 and Supply Chain and District Contracts (5th call) project “White Wine Identity: new horizons for an integrated and sustainable development of the Italian white wine supply chain”, which was funded by the Ministry of Agriculture, Food Sovereignty and Forests. Grant number: PRI-1058; MASAF public call n.182458 22-04-2022, D.D. MASAF 15.11.2023 n. 633056-position n.62; D.M. n. 0264374 12.06.2024, D.M. n. 399082 28.07.2023, n. 633056 15.11.2023 and n. 559310 23.10.2024.

Declaration of competing interest

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

Data availability

Data will be made available on request.

References

- Apolinar-Valiente, R., Romero-Cascales, I., Williams, P., Gómez-Plaza, E., López-Roca, J. M., Ros-García, J. M., & Doco, T. (2014). Effect of winemaking techniques on polysaccharide composition of cabernet sauvignon, Syrah and Monastrell red wines. *Australian Journal of Grape and Wine Research*, 20(1), 62–71. <https://doi.org/10.1111/ajgw.12048>
- Ayestarán, B., Guadalupe, Z., & León, D. (2004). Quantification of major grape polysaccharides (Tempranillo v.) released by maceration enzymes during the fermentation process. *Analytica Chimica Acta*, 513(1), 29–39. <https://doi.org/10.1016/j.aca.2003.12.012>
- Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta*, 76(5), 965–977. <https://doi.org/10.1016/j.talanta.2008.05.019>
- Brandão, E., Silva, M. S., García-Estévez, I., Williams, P., Mateus, N., Doco, T., ... Soares, S. (2017). The role of wine polysaccharides on salivary protein-tannin interaction: A molecular approach. *Carbohydrate Polymers*, 177, 77–85. <https://doi.org/10.1016/j.carbpol.2017.08.075>
- Canalejo, D., Martínez-Lapuente, L., Ayestarán, B., Pérez-Magarino, S., & Guadalupe, Z. (2024). Potential use of grape and wine polysaccharide extracts as fining agents to modulate the volatile composition of Viura wines. *Food Chemistry*, 430, Article 137047. <https://doi.org/10.1016/j.foodchem.2023.137047>
- Chambers, R. E., & Clamp, J. R. (1971). An assessment of Methanolysis and other factors used in the analysis of carbohydrate-containing materials. *The Biochemical Journal*, 125(4), 1009–1018. <https://doi.org/10.1042/bj1251009>
- Chaplin, M. F. (1982). A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas-liquid chromatography. *Analytical Biochemistry*, 123(2), 336–341. [https://doi.org/10.1016/0003-2697\(82\)90455-9](https://doi.org/10.1016/0003-2697(82)90455-9)
- Doco, T., O'Neill, M. A., & Pellerin, P. (2001). Determination of the neutral and acidic glycosyl-residue compositions of plant polysaccharides by GC-EL-MS analysis of the trimethylsilyl methyl glycoside derivatives. *Carbohydrate Polymers*, 46(3), 249–259. [https://doi.org/10.1016/S0144-8617\(00\)00328-3](https://doi.org/10.1016/S0144-8617(00)00328-3)
- Doco, T., Quéllec, N., Moutounet, M., & Pellerin, P. (1999). Polysaccharide patterns during the aging of Carignan noir red wines. *American Journal of Enology and Viticulture*, 50(1), 25–32. <https://doi.org/10.5344/ajev.1999.50.1.25>
- Doco, T., Williams, P., Pauly, M., O'Neill, M. A., & Pellerin, P. (2003). Polysaccharides from grape berry cell walls. Part II. Structural characterization of the xyloglucan polysaccharides. *Carbohydrate Polymers*, 53(3), 253–261. [https://doi.org/10.1016/S0144-8617\(03\)00072-9](https://doi.org/10.1016/S0144-8617(03)00072-9)
- Dufour, C., & Bayonove, C. L. (1999). Influence of wine structurally different polysaccharides on the volatility of aroma substances in a model system. *Journal of Agricultural and Food Chemistry*, 47(2), 671–677. <https://doi.org/10.1021/jf9801062>
- Dupin, I. V. S., McKinnon, B. M., Ryan, C., Boulay, M., Markides, A. J., Jones, G. P., ... Waters, E. J. (2000). Saccharomyces cerevisiae mannoproteins that protect wine from protein haze: Their release during fermentation and lees contact and a proposal for their mechanism of action. *Journal of Agricultural and Food Chemistry*, 48(8), 3098–3105. <https://doi.org/10.1021/jf0002443>
- Escot, S., Feuillat, M., Dulau, L., & Charpentier, C. (2001). Release of polysaccharides by yeasts and the influence of released polysaccharides on colour stability and wine astringency. *Australian Journal of Grape and Wine Research*, 7(3), 153–159. <https://doi.org/10.1111/j.1755-0238.2001.tb00204.x>
- Gerbaud, V., Gabas, N., C., L., Blouin, J. M., Vidal, S., Moutounet, M., & Pellerin, P. (1996). Effect of wine polysaccharides on the nucleation of potassium hydrogen tartrate in model solutions. *Chemical Engineering Research and Design*, 74(A7), 782–790.
- González-Royo, E., Urtasun, A., Gil, M., Kontoudakis, N., Esteruelas, M., Fort, F., ... Zamora, F. (2013). Effect of yeast strain and supplementation with inactive yeast

- during alcoholic fermentation on wine polysaccharides. *American Journal of Enology and Viticulture*, 64(2), 268–273. <https://doi.org/10.5344/ajev.2012.12071>
- Guadalupe, Z., & Ayestarán, B. (2007). Polysaccharide profile and content during the vinification and aging of tempranillo red wines. *Journal of Agricultural and Food Chemistry*, 55(26), 10720–10728. <https://doi.org/10.1021/jf0716782>
- Guadalupe, Z., & Ayestarán, B. (2008). Effect of commercial mannoprotein addition on polysaccharide, polyphenolic, and color composition in red wines. *Journal of Agricultural and Food Chemistry*, 56(19), 9022–9029. <https://doi.org/10.1021/jf801535k>
- Guadalupe, Z., Martínez-Pinilla, O., Garrido, Á., Carrillo, J. D., & Ayestarán, B. (2012). Quantitative determination of wine polysaccharides by gas chromatography-mass spectrometry (GC-MS) and size exclusion chromatography (SEC). *Food Chemistry*, 131(1), 367–374. <https://doi.org/10.1016/j.foodchem.2011.08.049>
- Jones-Moore, H. R., Jelley, R. E., Marangon, M., & Fedrizzi, B. (2021). The polysaccharides of winemaking: From grape to wine. *Trends in Food Science and Technology*, 111, 731–740. <https://doi.org/10.1016/j.tifs.2021.03.019>
- Jones-Moore, H. R., Jelley, R. E., Marangon, M., & Fedrizzi, B. (2022). The interactions of wine polysaccharides with aroma compounds, tannins, and proteins, and their importance to winemaking. *Food Hydrocolloids*, 123, Article 107150. <https://doi.org/10.1016/j.foodhyd.2021.107150>
- Li, S., Wilkinson, K. L., Mierczynska-Vasilev, A., & Bindon, K. A. (2019). Applying nanoparticle tracking analysis to characterize the polydispersity of aggregates resulting from tannin–polysaccharide interactions in wine-like media. *Molecules*, 24(11), 2100. <https://doi.org/10.3390/molecules24112100>
- Lubbers, S., Charpentier, C., Feuillat, M., & Voilley, A. (1994). Influence of yeast walls on the behavior of aroma compounds in a model wine. *American Journal of Enology and Viticulture*, 45(1), 29–33. <https://doi.org/10.5344/ajev.1994.45.1.29>
- Manjón, E., Recio-Torrado, A., Ramos-Pineda, A. M., García-Estévez, I., & Escribano-Bailón, M. T. (2021). Effect of different yeast mannoproteins on the interaction between wine flavanols and salivary proteins. *Food Research International*, 143, Article 110279. <https://doi.org/10.1016/j.foodres.2021.110279>
- Marangon, M., Marassi, V., Mattivi, F., Marangon, C. M., Moio, L., Roda, B., ... Curioni, A. (2025). The role of protein-phenolic interactions in the formation of red wine colloidal particles. *OENO one*, 59(2), Article 8485. <https://doi.org/10.20870/oeno-one.2025.59.2.8485>
- Mitropoulou, A., Hatzidimitriou, E., & Paraskevopoulou, A. (2011). Aroma release of a model wine solution as influenced by the presence of non-volatile components. Effect of commercial tannin extracts, polysaccharides and artificial saliva. *Food Research International*, 44(5), 1561–1570. <https://doi.org/10.1016/j.foodres.2011.04.023>
- Moine-Ledoux, V., & Dubourdieu, D. (1999). An invertase fragment responsible for improving the protein stability of dry white wines. *Journal of the Science of Food and Agriculture*, 79(4), 537–543. [https://doi.org/10.1002/\(SICI\)1097-0010\(19990315\)79:4<537::AID-JSFA214>3.0.CO;2-B](https://doi.org/10.1002/(SICI)1097-0010(19990315)79:4<537::AID-JSFA214>3.0.CO;2-B)
- Pellerin, P., Doco, T., Vidal, S., Williams, P., Brillouet, J.-M., & O'Neill, M. A. (1996). Structural characterization of red wine rhamnogalacturonan II. *Carbohydrate Research*, 290(2), 183–197. [https://doi.org/10.1016/0008-6215\(96\)00139-5](https://doi.org/10.1016/0008-6215(96)00139-5)
- Pellerin, P., Waters, E., & Brillouet, J.-M. (1993). Characterization of two arabinogalactan-proteins from red wine. *Carbohydrate Polymers*, 22(3), 187–192. [https://doi.org/10.1016/0144-8617\(93\)90139-U](https://doi.org/10.1016/0144-8617(93)90139-U)
- Riou, V.Á., Vernhet, A., Doco, T., & Moutounet, M. (2002). Aggregation of grape seed tannins in model wine-effect of wine polysaccharides. *Food Hydrocolloid*, 16, 17–23. [https://doi.org/10.1016/S0268-005X\(01\)00034-0](https://doi.org/10.1016/S0268-005X(01)00034-0)
- Rodrigues, A., Ricardo-Da-Silva, J. M., Lucas, C., & Laureano, O. (2012). Effect of commercial mannoproteins on wine colour and tannins stability. *Food Chemistry*, 131(3), 907–914. <https://doi.org/10.1016/j.foodchem.2011.09.075>
- Vidal, S., Doco, T., Moutounet, M., & Pellerin, P. (2000). Soluble polysaccharide content at initial time of experimental must preparation. *American Journal of Enology and Viticulture*, 51(2), 115–121. <https://doi.org/10.5344/ajev.2000.51.2.115>
- Vidal, S., Williams, P., Doco, T., Moutounet, M., & Pellerin, P. (2003). The polysaccharides of red wine: Total fractionation and characterization. *Carbohydrate Polymers*, 54(4), 439–447. [https://doi.org/10.1016/S0144-8617\(03\)00152-8](https://doi.org/10.1016/S0144-8617(03)00152-8)
- Vidal, S., Williams, P., O'Neill, M. A., & Pellerin, P. (2001). Polysaccharides from grape berry cell walls. Part I: Tissue distribution and structural characterization of the pectic polysaccharides. *Carbohydrate Polymers*, 45(4), 315–323. [https://doi.org/10.1016/S0144-8617\(00\)00285-X](https://doi.org/10.1016/S0144-8617(00)00285-X)
- Waters, E., Pellerin, P., & Brillouet, J. M. (1994b). A wine arabinogalactan-protein that reduces heat-induced wine protein haze. *Bioscience, Biotechnology, and Biochemistry*, 58(1), 43–48. <https://doi.org/10.1271/bbb.58.43>
- Waters, E. J., Pellerin, P., & Brillouet, J.-M. (1994a). A Saccharomyces mannoprotein that protects wine from protein haze. *Carbohydrate Polymers*, 23(1), 185–191. [https://doi.org/10.1016/0144-8617\(94\)90101-5](https://doi.org/10.1016/0144-8617(94)90101-5)
- Zhai, H. Y., Li, S. Y., Zhao, X., Lan, Y. B., Zhang, X. K., Shi, Y., & Duan, C. Q. (2023). The compositional characteristics, influencing factors, effects on wine quality and relevant analytical methods of wine polysaccharides: A review. *Food Chemistry*, 403, Article 134467. <https://doi.org/10.1016/j.foodchem.2022.134467>