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Improved size-exclusion high-performance liquid chromatographic method for the simple analysis of grape juice and wine polysaccharides

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Abstract

A size-exclusion HPLC method was developed for the separation and quantification of grape juice and wine polysaccharides. Polysaccharide extraction was carried out by precipitation and two chromatographic procedures were tested with the polysaccharide precipitate solution: direct injection, and fractionation of non-negatively charged (NCPS) and negatively charged (CPS) polysaccharides using an anion-exchange cartridge, before injection. The direct procedure (injection of the precipitate solution on two GPC columns and RI detection) was chosen, because the sum of areas of NCPS fractions represented 1.42% of the total area (sum of areas of NCPSs plus sum of areas of CPSs). The precision, linearity, sensitivity and accuracy of this method were established. Identification was performed by pectins and polyacrylic acids. The concentration of each polysaccharide identified according to the molecular mass was obtained by considering that the whole area (100%) contains the total number of polysaccharides determined by colorimetric method. Three polysaccharides were separated in grape juice (19 000; 2000 and 1000, approximately), whereas seven polysaccharides were obtained in wine (45 000; 19 000; 12 000; 7000; 4000; 2000 and 1000, approximately).

Keywords: Grape juice; Wine; Polysaccharides

1. Introduction

Grape juice and wine polysaccharides are the subject of a number of studies, since they have an important influence on several stages of the winemaking process such as: grape juice racking, fermentation, wine filtration and wine stabilization [1–6]. Moreover, these compounds affect the organoleptic properties of wines, including flavor [7], color [2,8] and foam [9–11]. Some authors have even identified the importance of the type of polysaccharide on such wine characteristics as flavor [12] and foaming [13].

Various methods are available for the study of polysaccharides in grape juices and wines. Some of these use colorimetric techniques [14–20]. The most recent spectrophotometric methods determine, not only the total polysaccharide content, but also acidic and neutral polysaccharide concentrations.

Other methods are size-exclusion chromatography

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(SEC) techniques that separate wine polysaccharides according to their molecular mass [21–23]. In this way, several authors have achieved a previous fractionation of wine polysaccharides according to their acidic–neutral properties by ion-exchange chromatography (IEC) [22,23]. To determine the concentration of the polysaccharides separated by these methods, these authors apply gas chromatography (GC) techniques to determine the monosaccharide composition of the polysaccharide fraction, after hydrolysis [24] and alditol acetate derivatization [25]. These methods are long, laborious and a considerable sample quantity is needed to carry out the whole process.

This study proposes a simplified method by which to identify and quantify the polysaccharide profile of grape juice and wine according to their approximate molecular mass. The method applies gel-permeation chromatography (GPC) and detection by refractive index (RI). Polysaccharides were extracted by precipitation, which was then used to test two procedures: direct injection on size-exclusion columns of the precipitate solution; and fractionation of neutral and acidic polysaccharides with an anion-exchange cartridge, before GPC analysis. Thus, we were able to compare the chromatographic profile of the total polysaccharide precipitate with the respective profiles of its acidic and neutral polysaccharide fractions obtained by anion-exchange. The distinction between acidic and neutral polysaccharides is of interest because it identifies the source of each type of wine polysaccharide: acidic polysaccharides are derived from the grape; whereas neutral polysaccharides are derived both from the grape and from the release from yeast during vinification [26]. The total soluble protein content of the polysaccharide precipitate and of each fraction was determined, because Segarra et al. [20] noted that 1% of the precipitate was constituted by proteins. Furthermore, in order to determine the extent of this interference, an UV detector was coupled in parallel with a RI detector.

The method proposed is simple and allows the polysaccharide profiles in the grape juice and wine of different grape varieties to be rapidly compared. Moreover, it offers the possibility of determining the extent to which the year of harvest influences on polysaccharide content, as well as determining which types of polysaccharide undergo changes during the winemaking process.

This method was applied to grape juice and wine obtained from this juice. The validation parameters were established.

2. Experimental

2.1. Chemicals

Standards of different molecular mass were used to perform the calibration curves. Dextrans (produced by *Leuconostoc mesenteroides*) (Sigma) of molecular mass (M_r) 11 000, 39 000, 71 000, 150 000, 267 000, 580 000. Pectins (potassium salt; from citrus fruit) (Sigma) of M_r 17 000, 34 000, 82 000 and 31, 93, 67% degree of esterification, respectively. Polyacrylic acids (Aldrich) of M_r 2000, 5100 (sodium salt), 8000 (sodium salt), 450 000. Each of these standards was separately injected at 0.025, 0.050 and 0.100 mg/ml (in double-distilled water). The polyacrylic acid of M_r 450 000 was not included in the calibration curves; it was used to compare its retention time with the retention time of the dextran of M_r 580 000.

A pectin solution (M_r 17 000; 0.2 mg/ml in double-distilled water) was prepared to determine the accuracy of the method.

The following solutions were used to perform the colorimetric determinations. For acidic polysaccharide determination: *o*-hydroxydiphenyl reagent at 0.15% (w/v) in 0.5% (w/v) sodium hydroxide, D-α-galacturonic acid (Merck) at 1 g/l (in double-distilled water) and sodium tetraborate (0.0125 *M*; in concentrated sulfuric acid) were used. For total polysaccharide determination: phenol reagent at 5% (w/v) (in double-distilled water) and D-(+)-galactose (Merck) at 1 g/l (in double-distilled water) were employed. To determine the concentration of soluble protein: bovine serum albumin (fraction V; from bovine blood) (Merck) at 100 mg/l (in double-distilled water) and Comassinne Blue protein reagent (Bio-Rad) were used.

2.2. Equipment

A Merck–Hitachi liquid chromatograph (655A-12;

L-5000 LC controller) equipped with two serial gel permeation columns was used. The loop sample injector had a capacity of 100 μl. Two detectors were used in parallel: a RI 1047A Hewlett-Packard and UV L-4200 Merck–Hitachi. An integrator L-2000 (Merck–Hitachi) was connected to each detector.

A Hewlett-Packard diode array spectrophotometer 8452A in combination with Hewlett-Packard 89531A UV–Vis operating software was used.

2.3. Conditions

2.3.1. Preparation of samples

White grape juice and wine samples were provided by Freixenet SA Winery. Both samples were centrifuged at 2500 *g* for 20 min at 4°C and were kept in the freezer (−18°C) until analysis.

2.3.2. Polysaccharide precipitation

Ethanol (96%, v/v) and HCl (1 *M*) were added to 4 ml of sample (grape juice and wine) (proportion of ethanol, 5:1; proportion of HCl, 50 ml/l). The dried precipitates of grape juice and wine were dissolved with 8 ml or 2 ml of double-distilled water, respectively.

This extraction was performed in duplicate on each sample.

2.3.3. Separation between non-negatively charged (NCPS) and negatively charged (CPS) polysaccharides by anion-exchange chromatography

Total polysaccharide extract was fractionated by anion-exchange chromatography, with a Sep-Pak cartridge (Waters Accell Plus QMA), equilibrated with 10 ml of double-distilled water. The non-negative-charged polysaccharides (NCPSs) were recovered just after sample application since they were not retained by the cartridge. One ml of double-distilled water was eluted to secure the total recovery of NCPSs. The negative-charged polysaccharides (CPSs) were recovered by applying 6 ml of KCl (0.4 *M*). These solvents and volumes were chosen since the total elution of the wine polysaccharides could be verified.

2.3.4. Colorimetric methods

Total, acidic and neutral polysaccharide contents were determined following the method described by Segarra et al. [20], while the soluble protein content was quantified according to the Bradford method [27]. These determinations were performed on the polysaccharide precipitate of the grape juice and wine and on the NCPS and CPS fractions of the wine polysaccharides (Table 2).

2.3.5. Gel-permeation chromatography

The mobile phase was 0.1 *M* KCl and was used at a flow-rate of 0.3 ml/min. Both serial columns were Ultrahydrogel 250 (30 cm×7.8 mm I.D.). A guard column PW_{XL} (4.0 cm×6.0 mm I.D.) was also used. These columns were maintained at 40°C. Polysaccharide precipitate solution, NCPS and CPS fractions were injected (100 μl) after filtration through a 0.2-μm nylon membrane.

2.4. Quantification

The absolute response factor of the dextran of M_r 150 000, the pectin of M_r 34 000 and the polyacrylic acid of M_r 8000 were chosen to obtain the relative response factors of dextrans, pectins and polyacrylic acids, respectively (Table 1).

The % area of each polysaccharide identified according to its molecular mass, was obtained by internal normalization. An area of 100% corresponded to the concentration of total polysaccharides obtained by the colorimetric method. Therefore, the amount of each polysaccharide could be calculated from these data (Table 3).

2.5. Validation of proposed method

2.5.1. Precision

Repeatability was expressed as coefficient of variation of the mean area counts, and also as the standard deviation of the mean amounts of polysaccharides separated by GPC. Three precipitate solutions of each sample (grape juice and wine) were injected in duplicate ($n=6$).

These six injections were used to determine the retention time repeatability, which was expressed as the confidence interval (Table 3).

Table 1
Retention time means and relative response factors of the standards and calibration curve equations

	M_r	t_R mean[a] (min)	Relative response factor
Dextrans (Ds)	D – 580 000	37.08	1.10
	D – 267 000	37.27	1.07
	D – 150 000[b]	39.90	1.00
$y=1E+24x^{-11.78}$	D – 71 000	41.36	1.04
	D – 39 000	43.61	1.02
	D – 11 000	49.29	1.14
Polyacrylic acids (PACs)	PAC – 450 000[e]	35.70	0.93
and	PEC – 82 000	36.90	1.02
pectins (PECs)	PEC – 34 000[c]	37.74	1.00
	PEC – 17 000	38.50	0.95
$y=5E+18x^{-8.9037}$	PAC – 8000[d]	47.71	1.00
	PAC – 5100	49.43	0.90
	PAC – 2000	51.46	1.00

x=Retention time (min).
y=Molecular mass.
[a] Mean was obtained with $n=6$.
[b] Reference standard for response factors of dextrans (Ds)
[c] Reference standard for response factors of pectins (PECs).
[d] Reference standard for response factors of polyacrylic acids (PACs).
[e] This standard was not included in the calibration curve.

2.5.2. Accuracy

Five hundred microliters of the pectin solution (0.2 g/l) were added to 500 μl of the solution of the wine polysaccharide precipitate in quintuplicate. The accuracy was expressed as the recovery percentage of the pectin solution added.

2.5.3. Sensitivity and linearity

The wine polysaccharide precipitate was dissolved in 2 ml of water in triplicate. From this solution, three dilutions were prepared (0.5:10; 1.5:10; 3:10). These four solutions were injected in duplicate ($n=24$). The regression curves of each polysaccharide separated according to their molecular mass, were obtained. The correlation coefficients, slopes, intercepts and standard deviations of the curves were used to determine the linearity, detection and quantification limits [28] (Table 4).

3. Results and discussion

Table 1 shows the retention time means of the standards of different molecular mass, and the calibration curve equations obtained with them. We considered it necessary to perform two calibrations, one with dextrans and other with pectins and polyacrylic acids, because the anionic compounds in these size-exclusion columns eluted earlier than theoretically expected since ionic interactions were also found to occur. In order to eliminate these interactions a fluent with a greater ionic strength can be used (for instance, 0.01–0.1 M $NaNO_3$) [29]. However, although several salts at different concentrations were tested, differences were recorded between the retention times of charged standards and those of neutral standards of similar molecular mass (Table 1).

The relative response factors of the standard compounds (Table 1) showed that the specific refractive index increment (dn/dc) was independent of the molecular mass and constant in the molecular mass range studied. Moreover, the response of the grape juice and wine polysaccharides, which was expressed as the slope of the respective regression curve (concentration vs. area) (Table 4), was also similar between them. Therefore, we consider that neither the structure nor the molecular mass did not in-

fluence on the refractive index response of the polysaccharides. So that, we can quantify the grape juice and wine polysaccharides by internal normalization.

Fig. 1a Fig. 1b show the GPC chromatographic profiles of the NCPS and CPS fractions of the wine obtained by the anionic exchange chromatography. It was observed that the area of the NCPS chromatogram was much lower than that of the CPS chromatogram. Moreover, neutral and acidic polysaccharide content in these fractions – obtained by the colorimetric methods – were different to the content of these two types of polysaccharide in the total precipitate (Table 2). This might be due to the fact that the cartridge separated the negatively charged polysaccharides from the other polysaccharides, whereas the colorimetric method only determined the acidic polysaccharides according to the content of

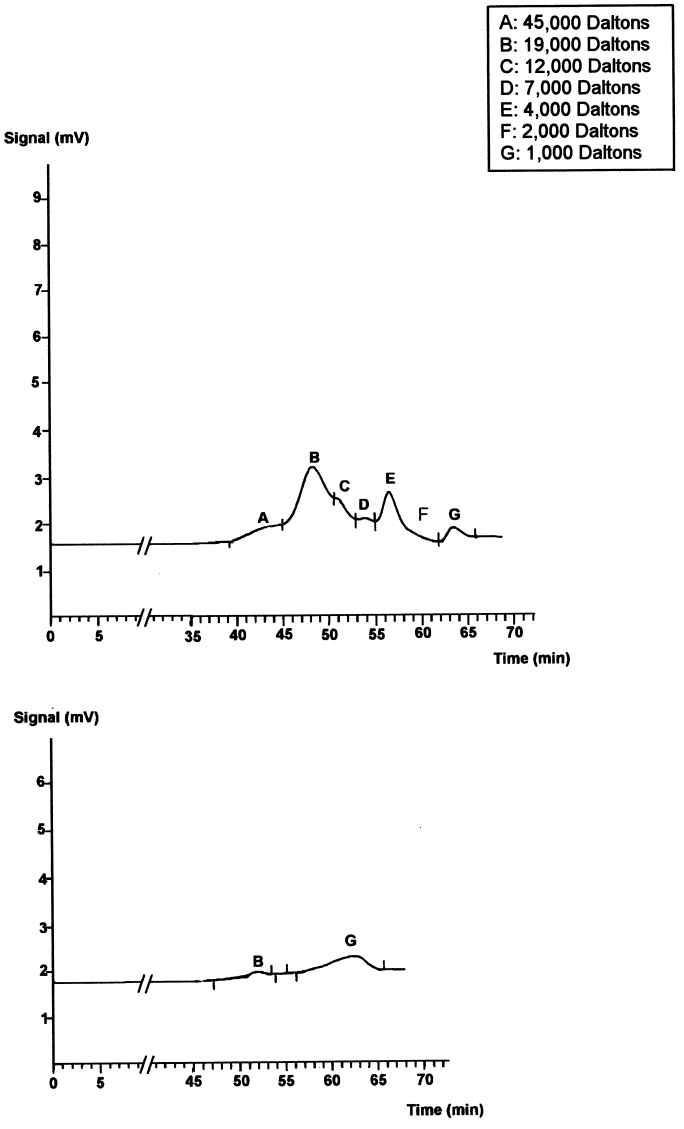

Fig. 1. (a) Chromatogram of the non-negatively charged polysaccharide (NCPS) fraction of the wine. (b) Chromatogram of the negatively charged polysaccharide (CPS) fraction of the wine.

Table 2
Results of the polysaccharide and protein contents of wine precipitate and NCPS and CPS fractions, determined by spectrophotometry

	TPS[a] (Mean±S.D.) (n=2)	APS[b] (Mean±S.D.) (n=2)	NPS[c] (Mean±S.D.) (n=2)	Proteins[d] (Mean±S.D.) (n=2)
Wine PS precipitate	340±1	58±1	282±1	1.3±0.06
Wine NCPS[e]	22±3	<LOQ	20±3	N.D.
Wine CPS[f]	359±36	37±6	322±40	N.D.

[a] TPS: concentration of total polysaccharides (mg galactose/l).
[b] APS: concentration of acid polysaccharides (mg galacturonic acid/l).
[c] NPS: concentration of neutral polysaccharides (mg galactose/l).
[d] Results expressed as mg albumin/l.
[e] Wine NCPS: non-negatively charged polysaccharides of wine precipitate.
[f] Wine CPS: negatively charged polysaccharides of wine precipitate.

galacturonic acid, since the chromogen reagent reacts specifically with this compound. Furthermore, it was observed that the acidic polysaccharide content of the NCPSs – obtained with the colorimetric method – was inferior to the limit of quantification for this method (2.2 mg/ml). For this reason, we considered all wine polysaccharides constituted by galacturonic acid (pectins) as having been retained on the anionic cartridge.

The NCPS fraction should not absorb at UV. Nevertheless, the wine NCPS fraction gave absorbance in the UV detector. This might have been due to the presence of proteins in this fraction. The wine polysaccharide precipitate had 1.3 mg/l of protein (Table 2). Moreover, after the anion-exchange fractionation, it is probable that these proteins were in the NCPS fraction, because the precipitate was dissolved with double-distilled water whose pH was higher than the isoelectric point of wine proteins (3.5–4.5, according to Brissonnet and Maujean [30]). However, it was not possible to corroborate the protein content in the NCPS fraction, because the Bradford colorimetric method [27] was not sensitive enough.

The profile of the CPS fraction was very similar to the profile obtained when the total precipitate solution was directly injected (Fig. 1a Fig. 2b). Furthermore, the sum of areas of NCPS fraction represented 1.42% of the total area (sum of areas of NCPSs plus sum of areas of CPSs) (Fig. 1b Fig. 2b). Consequently, most wine neutral polysaccharides behaved as negatively charged polysaccharides, and were retained by the cartridge. This result could justify the high neutral polysaccharide content obtained after the colorimetric method was applied to the CPS fraction (Table 2). Moreover, other authors [23] also consider that most wine polysaccharides behave as acids, given the presence not only of uronic acids (characteristic of pectins) but also of phosphorus.

Considering these results, it was decided not to perform the first separation of the two fractions (NCPS and CPS) by anionic exchange with the cartridge, but rather the direct injection of the solution of the grape juice or wine polysaccharide precipitate. Furthermore, as we considered most of the polysaccharides to have negative charges, we only used the calibration obtained with the negatively charged compounds (Table 1).

If the two polysaccharide fractions (NCPSs and CPSs) were not separated, it would be impossible to erase the interference of the proteins, because most of grape juice and wine polysaccharides were charged and, consequently, they also absorb at UV. Nevertheless, this error could be accepted because the polysaccharide precipitate had a very low content of proteins (Table 2). Moreover, the sum of areas of the NCPS fraction (proteins were likely to be found here) represented a very low percentage (1.42%, as we noted above) of the total area (sum of areas of NCPSs plus sum of areas of CPSs) (Fig. 1b Fig. 2b). In addition, the proteins constitute part of the structure of some types of grape juice and wine polysaccharides: mannoproteins and arabinogalactan-proteins [31], and so, the molecular size of these polysaccharides was influenced by the proteins.

The chromatograms of the grape juice and wine polysaccharide precipitates are shown in Fig. 2a Fig. 2b. Table 3 contains the molecular mass, the % area

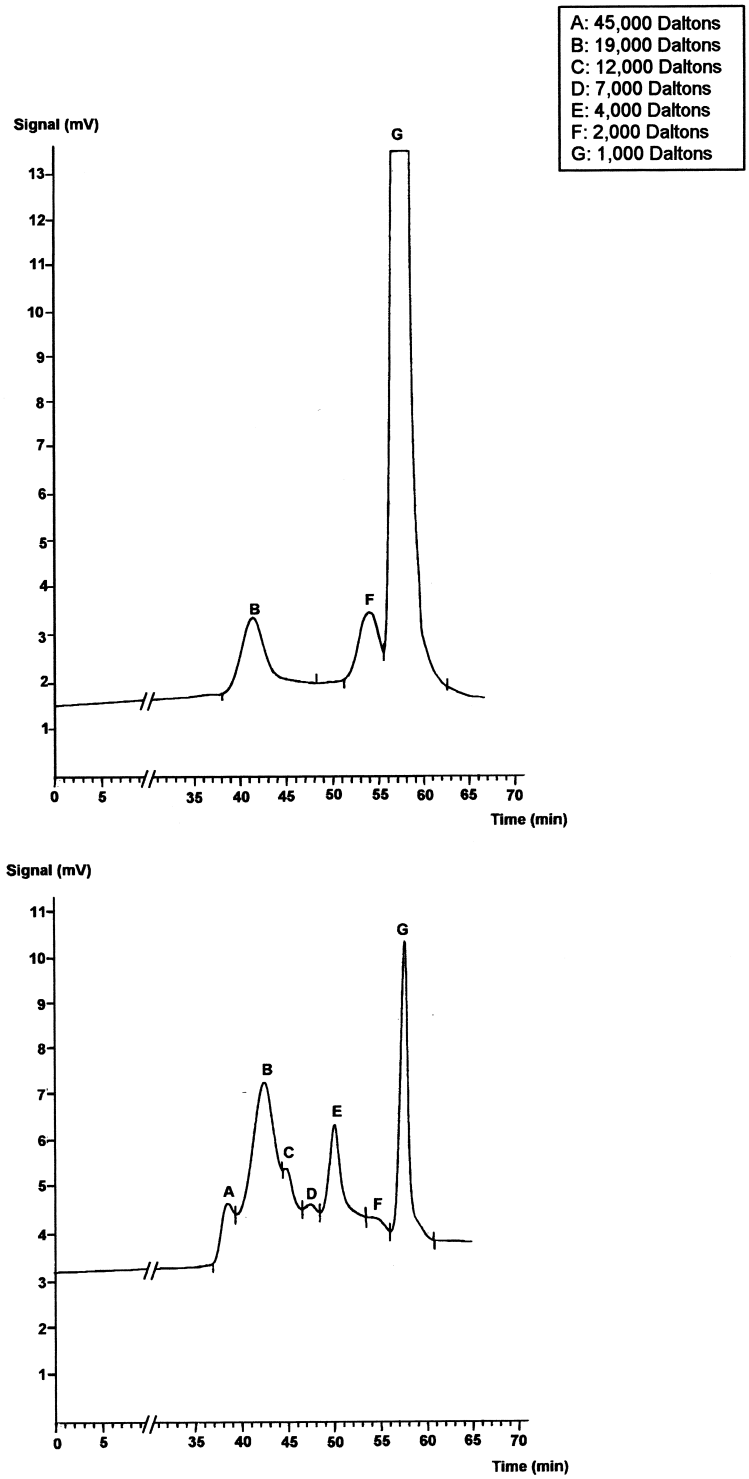

Fig. 2. (a) Chromatogram of grape juice polysaccharides. (b) Chromatogram of wine polysaccharides.

Table 3
Repeatability, identification and quantification of grape juice and wine polysaccharides

	Retention time repeatability [IC[a] of t_R (min)]	Identification (M_r)	Quantification and method repeatability		
			% Area account mean[c]	C.V.[b] (%)	Amount mean (mg/l)[d]
Grape juice	41.80–41.34	18 000–20 000	3.61	0.22	14±2
	54.14–53.75	2000–3000	2.13	0.88	8±1
	57.46–56.75	1000–2000	94.26	4.85	350±3
Wine	37.51–37.10	42 000–47 000	7.63	3.59	26±3
	41.45–40.98	17 000–20 000	37.31	2.60	127±4
	44.04–42.80	11 000–13 000	9.29	2.84	32±1
	46.54–46.00	6000–7000	5.10	6.20	17±1
	49.20–48.59	3000–4000	16.73	4.27	57±4
	53.68–51.11	2000–3000	3.75	11.31	13±5
	56.60–56.12	1000–2000	20.19	3.38	69±7

[a] Confidence interval of the mean retention time; mean was obtained with $n=6$.
[b] Coefficient of variation of the mean area count.
[c] Mean was obtained with $n=6$.
[d] Results expressed as mg galactose/l.

and the amount of each polysaccharide separated by GPC. It can be observed that the three types of grape juice polysaccharide (one of M_r 20 000–18 000, other of M_r 2000–3000 and the other of M_r 1000–2000) were also in the wine. The other polysaccharides of the wine might be the polysaccharides released by yeast during alcoholic fermentation.

The quantification of polysaccharides had a variation coefficient lower than 5% for most of the polysaccharides separated according to their molecular mass, with the exception of the wine polysaccharides of M_r 6000–7000 and 2000–3000 (Table 3). This could be due to the lower area percent of these peaks. The retention time repeatability was correct for all polysaccharides, since the confidence intervals of the retention times were narrow and did not overlap (Table 3).

The recovery was 94.1±5.1% (expressed as mean±S.D., $n=5$). This recovery can be considered valuable, since the gel permeation chromatography has a poor resolution.

The regression curves of each polysaccharide separated by GPC showed a satisfactory linearity [$r \geq 0.9992$, with the exception of the polysaccharide of M_r 2000–3000 ($r=0.9966$)] in the range in which the curves were established (Table 4). The detection

Table 4
Sensitivity, linearity and detection and quantification limits of the wine polysaccharides

Wine polysaccharides (M_r)	Linearity (r)	Sensitivity		DL (mg/l)	QL (mg/l)
		Intercept (a)	Slope (b)		
42 000–47 000	0.9994	−4913	5584	3	10
17 000–20 000	0.9992	−24 481	5578	16	32
11 000–13 000	0.9994	−6785	5599	4	12
6000–7000	0.9993	−3386	5587	2	7
3000–4000	0.9996	−9124	5594	6	17
2000–3000	0.9966	−3372	5571	2	7
1000–2000	0.9994	−13 588	5585	8	27

and quantification limits (Table 4) allowed to determine the concentration of the polysaccharides of different molecular mass at the ppm level.

As the peaks obtained by SEC were not completely resoluted, these results were as expected. Thus, we concluded that the method was adequate for the analysis of grape juice and wine polysaccharides according to their molecular mass.

4. Conclusions

Here, we have proposed a simplified method for determining the grape juice and wine polysaccharides. The validation parameters (precision, linearity, sensitivity and accuracy) showed that the method was adequate for the analysis of grape juice and wine polysaccharide according to their molecular mass.

The fractionation of wine polysaccharides using a cartridge of anion-exchange was tested, obtaining two fractions: NCPS and CPS polysaccharides. It was observed that the sum of areas of the NCPS fraction only represents 1.42% of the total area of the wine polysaccharide precipitate. Consequently, the grape juice and wine polysaccharides were mainly negatively charged polysaccharides. Moreover, these two fractions (NCPSs and CPSs) did not correspond to the neutral and acidic polysaccharides obtained by the colorimetric method. This could be due to the colorimetric method specifically determines the uronic acids and, consequently, the acidic polysaccharides were only pectins, whereas most of named neutral wine polysaccharides – obtained by colorimetric method – were charged.

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