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Effects of combining high power ultrasound and enological enzymes on the composition of polysaccharides in red wine

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ABSTRACT

In this work different oenological techniques, used at semi-industrial scale, were applied for their effect of deconstructing the polysaccharide network of the cells of the grape during the maceration: high-power ultrasound (US), a new technique recently introduced in the wine industry, and other conventional ones, such as the addition of pectolytic enzymes (E). The objective was to study if the combined effect of US and E, used at the moment of crushing, had a synergistic effect on the content of polysaccharides in red wines, compared to red wines made with the techniques applied separately. The timing of enzyme addition, maceration time and the ripening state of the grapes were the studied variables. Ultrasound treatment showed a greater effect than enological enzymes when used alone, especially when the ripest grapes were employed. The results also indicated that the combined treatment, regardless of the order of enzyme addition, did not improve the release of grape polysaccharides into the wine. Sonication maintained the same profile of polysaccharides than the control wine. The study demonstrated that sonication treatment increased the content of polysaccharides from the grapes into the wines, allowing a reduction of the maceration time.

1. Introduction

Major wine polysaccharides from the pectocellulosic portion of the cell walls of grapes are polysaccharides rich in arabinose and galactose (PRAG) (arabinogalactans type I, AG-I, and arabinogalactans type II joined to protein, AGP), and rhamnogalacturonans (rhamnogalacturonans type I, RG-I, and rhamnogalacturonans type II, RG-II) and homogalacturonans (HL), in contrast to mannoproteins (MP) from yeast cell walls (Martínez-Lapuente, Guadalupe, & Ayestarán, 2019). Polysaccharides play a fundamental role on the wine physical-chemical properties, being AGP/PRAG and MP strong inhibitors of the aggregation of tannins and preventing formation of large colloids. Moreover, RG-II dimers form co-aggregates with tannins (Riou, Vernhet, Doco, & Moutounet, 2002), and precipitation of tannin-protein complexes is largely reduced in the existence of wine polysaccharides (Maury, Sarni-Manchado, Poinsaut, Cheynier, & Moutounet, 2016). All these properties will clearly affect wine mouthfeel as viscosity, astringency, and hotness of the wines. Polysaccharides can also interact with aroma compounds (Villamor, Evans, Mattinson, & Ross, 2013).

Several authors describe the pectocellulosic portion as one of the main constituents of the grape cell wall (Gao, Fangel, Willats, Vivier, & Moore, 2015; Osete-Alcaraz, Gómez-Plaza, Pérez-Porras, & Bautista-Ortín, 2022) and, in addition, the pectic families are the main polysaccharides in wine (Ducasse et al., 2010; Guadalupe & Ayestarán, 2007). There are several factors that modify the extractability of polysaccharides from the grape cell wall into the wine. The degree of ripening of the grape increases the extractability of polysaccharides (Gil et al., 2015; Martínez-Lapuente et al., 2016) and, with the same degree of ripening, the extractability of polysaccharides depends on the variety of grape used (Ortega-Regules, Ros-García, Bautista-Ortín, López-Roca, & Gómez-Plaza, 2008). Currently, the wine industry has the option of using innovative techniques, such as ultrasound (US), and other conventional ones, such as enzymes (E), to break the polysaccharide network of the cell, facilitating the release of favorable compounds during winemaking such as polysaccharides and polyphenols.

In red wine production, commercial cocktails of maceration enzymes formed by pectolytic enzymes (primarily polygalacturonase, and to a lesser degree, pectin methylesterase and pectin-lyase activities)

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combined with hemicellulases and/or cellulases (Kassara, Li, Smith, Blando, & Bindon, 2019; Romero-Cascales, Fernández-Fernández, Ros-García, López-Roca, & Gómez-Plaza, 2008), are used to improve the color and increase the tannin content in the wines. Regardless of the degree of improvement in the phenolic composition of the wine, pectolytic-based commercial enzymes cause a considerable degree of depectinization and unraveling of the cell walls of the skins of grape berries during maceration-fermentation (Zietsman et al., 2015) and even enzymatic maceration has a much stronger effect on grape cell wall structures than ripening (Kuhlman, Hansen, Jørgensen, du Toit, & Moore, 2022). Pectinase rich enzyme preparations led to a change of the molecular weight distribution of polysaccharides released into the wines; and enzyme-treated wines contained more RG-II and less PRAG over the three vintages (Ducasse et al., 2010). It has also been shown that the pectolytic-based commercial enzymes affected the colloidal properties of wines, potentially increasing polysaccharide solubility (Kassara et al., 2019).

Recently, the OIV (2019) has approved the industrial use of ultrasound (US) on crushed grapes, based on the positive effect on the extraction of compounds from the grape into the wine in less time of vinification (Celotti et al., 2021; Morata et al., 2021; Pérez-Porras, Bautista-Ortín, Jurado, & Gómez-Plaza, 2022). Studies of the use of high-power ultrasound in a semi-industrial scale on the crushed Monastrell grapes demonstrated the improvement of the chromatic characteristics and the increase in the content of tannins and anthocyanins of red wines (Pérez-Porras, Bautista-Ortín, Jurado, & Gómez-Plaza, 2021; Gómez-Plaza, Osete-Alcaraz, Jurado, Iniesta, & Bautista-Ortín, 2020; Osete-Alcaraz, Bautista-Ortín, Ortega-Regules, & Gómez-Plaza, 2019; Bautista-Ortín et al., 2017), better scores in the olfactory attributes of wines (Oliver Simancas et al., 2021) and an increase in the content of grape polysaccharides in the wines (Martínez-Lapuente, Guadalupe, Ayestarán, et al., 2021; Martínez-Lapuente, Guadalupe, Pérez-Porras, et al., 2021). All these improvements in Monastrell wines were obtained by reducing the maceration time from 7 days to 3 days and were caused by the mechanism of the acoustic cavitation of high-power US at 28 kHz. The sound waves and the collapsing cavitation bubbles may induce either one or combination of the phenomena such as localized erosion, pore formation, fragmentation, shear force, increased absorption, and swelling index in the cell walls of the plants (Kumara, Srivastava, & Sharanagat, 2021), and all these phenomena contribute to improve the extraction yield.

The goal of deconstructing the polysaccharide network of the cells in both US and E techniques is the same, so their combination could enhance the extractability and solubility of grape polysaccharides into the wine. In E-assisted US extraction, the order of enzyme addition can also influence the extraction and solubility of polysaccharides from grapes to wines (Bansode & Rathod, 2017). There are no studies in the literature of the combined effect of both techniques applied in a semi-industrial scale on crushed grapes at the beginning of maceration. Therefore, the objective of this research was to study, in a semi-industrial scale, if the combination of US and E at the beginning of maceration enhances the effect of any of the techniques applied by itself and whether the results are affected by the ripening state of the grapes. In the most immature stage of the grape, the effect of the order of addition of the E in the combination of both techniques is also studied.

2. Materials and methods

2.1. Vinification and sample collection

Red grapes from *Vitis vinifera* var. Monastrell (VIVC: 7915) were grown in Jumilla (Murcia, Spain), and were harvested on the vintage 2020 at two stages of ripeness (12 °Baumé and 14 °Baumé) (hand-refractometer, ATAGO, Tokyo, Japan). Grapes were taken to the small-scale winery in plastic boxes of 20 kg that were stored refrigerated (3 °C).

Grapes were destemmed and crushed (Nouva Zambelli, Saonara Padova, Italy), sulphited (70 mg SO_2/kg) and divided into six vinifications (with three repetitions) for each stage of ripeness. The wines obtained were: two controls (C) with neither enzymatic nor ultrasound treatment with 3 or 7 days of skin maceration (W12–C3d, W12–C7d, W14–C3d and WC14-7d); two treated with sonication (US) of the crushed-destemmed grape and with 3 or 7 days of maceration (W12-US3d, W12-US7d, W14-US3d and W14-US7d); two treated with a pectolytic enzyme (E) with 3 days of maceration (W12–C + E3d, W14–C + E3d); and two treated in combination US + E with 3 days of maceration, the addition of E was after US (W12-US + E3d, W14-US + E3d). The enzymatic and US treatment were the same as those described by Pérez-Porras et al. (2022). For the enzymatic treatments, a comercial pectolytic enzyme (EnozymLux®, Agrovin, S.A., Alcazar de San Juan, Spain) was added at the concentration recommended by the supplier (3 mL/hL). For the US treatment, the crushed grapes were treated with a pilot-scale power ultrasound system (Agrovin S.A., Alcazar de San Juan, Spain) using a frequency of 30 kHz, a power of 9000 W and a power density of 58.5 W/cm^2. The ultrasound system comprised two hexagonal sonoreactors with sonoplates arranged along the pipes. The ultrasound system worked with a low rate of 400 kg of grapes per hour. The temperature of the crushed grapes did not increase by more than 2 °C.

For the 12 Baumé grapes, another vinification was carried out adding the pectolytic enzyme, and immediately, these were sonicated (E + US3d wine), to determine the effect of the moment of enzyme addition on the outcome of the final wine (W12-E + US3d).

All vinifications were similar to those described by Pérez-Porras et al. (2022). The wines obtained were cold stabilized at 2 °C for one month and bottled. At this time, the wines obtained were analyzed.

2.2. Identification and quantification of monosaccharides and polysaccharides by GC–MS

Wine polysaccharides were recovered by precipitation after ethanolic dehydration as previously described (Ayestarán, Guadalupe, & León, 2004; Guadalupe, Martínez-Pinilla, Garrido, Carrillo, & Ayestarán, 2012). The monosaccharide composition was determined by GC–MS of their trimethylsilyl-ester *O*-methyl glycosyl residues obtained after acidic methanolysis and derivatization as previously described (Doco, Quellec, Moutounet, & Pellerin, 1999; Doco, Vuchot, Cheynier, & Moutounet, 2003; Guadalupe, Ayestarán, Williams, & Doco, 2015; Guadalupe et al., 2012). GC was controlled by ChemStation software and equipped with a 7653B automatic injector consisting of an Agilent 7890A gas chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a 5975C VL quadrupole mass detector (MS). The total monosaccharides components of the precipitated polysaccharides were called TMS and total monosaccharides components the precipitated polysaccharides without mannose and glucose were called TMSG. The content of total soluble polysaccharides families (TSP) was estimated from the sum of PRAG, MP, and RG-II (Ayestarán et al., 2004; Doco et al., 1999).

The content of MP, RG-II, and PRAG was calculated according to previously described (Canalejo, Guadalupe, Martínez-Lapuente, Ayestarán, & Pérez-Magariño, 2021). The known molar ratios of constituent monosaccharides were used for the calculation of each class of polysaccharide family. Three replicates of analysis were performed for each wine sample.

2.3. Statistical analyses

Analyses of variance (ANOVA) and multivariate analysis of variance (MANOVA) were performed using the SPSS v. 15.0 for Windows statistical package (SPSS Statistics, Inc., Chicago, IL, USA) program. Differences between means were compared using Duncan's test. $p < 0.05$ was considered statistically significant. Pillai's trace and Wilks Lambda were used as test statistics in MANOVA. The percentages of variance

attributable to each factor (ripening degree, sonication treatment and enzyme addition) were calculated from the ratio between the sum of squares of each factor and the total, multiplied by 100.

3. Results and discussion

3.1. Effect of ultrasound and enzyme treatments on the glycosyl residue composition of wines polysaccharides and on polysaccharides families of wines

The concentrations of the glycosyl residues of the polysaccharides of the red wines obtained with grapes with a degree of maturation of 12 °Baumé and 14 °Baumé are presented in Table 1 and Table 2, respectively. The polysaccharides found in the wines corresponded to the three families of polysaccharides: MP, PRAG and RG-II. Their content was estimated from the concentration of individual glycosyl residues distinctive of each family, and the hydrolysis yield was considered for the calculation, as described by Doco et al. (1999). RG-II dimer can form co-aggregates with tannins (Riou et al., 2002) and large colloids that precipitate in the wine, while PRAG are strong inhibitors of the aggregation of tannins and prevent the formation of large colloids. From a sensory perspective, Brandão et al. (2020) concluded that the RG-II acidic fraction more effectively modulates wine astringency than the AGP fraction, which is a neutral polysaccharide. These authors observed that the acid fraction of RG-II was especially effective in inhibiting the precipitation of acidic proline-rich proteins and tatherin and P–B peptide, constituents of saliba (Brandão et al., 2020), but in the presence of Na^+ ions in solution, no RG-II effect was observed on salivary proteins−tannin interactions.

The major glycosyl residues in wines (Tables 1 and 2) were galactose, mannose, arabinose and galacturonic acid. Galactose, arabinose, rhamnose and glucuronic acid are the components of the pectic polysaccharides rich in arabinose and galactose (PRAG), as galacturonans, galactans, arabinogalactans, arabinogalactan proteins and arabinans (Vidal et al., 2003). Mannose residues in the wines are attributed to mannoproteins (MP) from yeast cell walls (Guadalupe & Ayestarán, 2007; Martínez-Lapuente et al., 2018).

The minor glycosyl residues of the wines were rhamnose, fucose, glucuronic acid, 2-*O*-methyl-xylose, 2-*O*-methyl-fucose, apiose, and Kdo (Tables 1 and 2). The 2-*O*-methyl-xylose, 2-*O*-methyl-fucose, apiose, and Kdo are markers for the presence of the pectic polysaccharides RG-II (Pellerin et al., 1996; Pérez, Rodríguez-Carvajal, & Doco, 2003; Vidal et al., 2003). The concentration of rhamnose and fucose can come from the pectic polysaccharides RG-I or RG-II in the case of rhamnose (Martínez-Lapuente et al., 2018), or only from RG-II in the case of fucose (Pellerin et al., 1996). Other minor glycosyl residues in the wines were xylose and glucose. Xylose residues were also found, indicating that traces of hemicellulose had been solubilized from the grape cell walls (Doco et al., 1999). The detection of glucose, which is not a component of pectic polysaccharides, was attributed to polysaccharides of yeasts and/or bacteria (Apolinar-Valiente et al., 2014; Martínez-Lapuente, Guadalupe, Ayestarán, Ortega-Heras, & Pérez-Magariño, 2013).

Table 1 shows that the addition of enzymes to grapes with "more intact" cell walls due to their lower technological maturation (Garrido-Bañuelos et al., 2019) resulted in wines with three days of maceration having a significantly higher content of some of the minor sugars called "rare" (2-*O*MeFuc and Kdo), markers of the presence of RG-II, rhamnose, fucose and galacturonic acid; and a lower content of the main monosaccharides of the PRAG (galactose, and arabinose), and of xylose with respect to the control wines, W12–C3d. Therefore, the addition of pectolytic enzymes modified the composition of the polysaccharides released from the grape cell wall, reducing the concentration of PRAG and increasing the concentration of RG-II compared to control wines, W12–C3d (Table 1). These results agree with previous studies in the literature (Apolinar-Valiente et al., 2014; Doco, Williams, & Cheynier, 2007; Ducasse et al., 2010).

The sonication treatment (W12-US3d), however, caused a higher content of total grape monosaccharides (TMSG), and of arabinose and galactose compared to control wines (W12–C3d) and wines obtained with the addition of enzymes (W12–C + E3d), and a significant increase in the content of 2-*O*MeFuc and rhamnose with respect to wines W12–C3d (Table 1). This meant that PRAG + RG-II content of the sonicated wines was significantly higher than E-added and control wines (Table 1). Brandão et al. (2017) showed that PRAG and RG-II were able to reduce the interactions between salivary proteins and tannins. Therefore, sonication, by increasing the PRAG + RG-II content in the

Table 1
Monosaccharide composition (mg/L) of polysaccharides and polysaccharides families (mg/L) in wines from 12° Baumé grapes.

Compound (mg/L)	Samples						
	W12–C3d	W12–C + E3d	W12-US3d	W12-US + E3d	W12-E + US3d	W12–C7d	W12-US7d
2-*O*MeFuc	3.75 ± 0.64a	8.29 ± 0.68c	6.30 ± 1.14b	6.58 ± 0.77b	8.09 ± 0.69c	9.76 ± 0.62d	8.72 ± 0.54cd
2-*O*MeXyl	1.89 ± 0.44a	4.91 ± 0.91 ab	5.33 ± 3.11 ab	6.69 ± 3.79b	4.56 ± 0.31 ab	4.58 ± 0.45 ab	5.22 ± 0.85 ab
Api	0.98 ± 0.05 ab	1.40 ± 0.95b	1.21 ± 0.29 ab	0.37 ± 0.02a	1.52 ± 0.89bc	1.78 ± 0.36bc	2.43 ± 0.28c
Kdo	0.67 ± 0.18 ab	2.01 ± 0.78c	1.39 ± 0.62bc	0.36 ± 0.01a	3.15 ± 0.68d	1.24 ± 0.29bc	1.81 ± 0.08c
Ara	43.94 ± 2.13b	21.85 ± 0.66a	69.22 ± 2.82c	30.39 ± 11.75a	24.27 ± 6.74a	90.00 ± 3.87d	90.23 ± 4.52d
Gal	244.64 ± 7.38b	188.20 ± 5.21a	317.25 ± 35.62c	269.70 ± 33.11b	171.91 ± 10.73a	376.23 ± 17.74d	367.22 ± 23.42d
GalA	51.47 ± 8.10 ab	76.96 ± 9.73bc	70.63 ± 6.94bc	34.58 ± 10.22a	52.41 ± 11.98 ab	98.30 ± 39.88c	130.47 ± 10.78d
GluA	8.60 ± 0.57b	9.51 ± 4.10b	10.94 ± 3.41b	1.99 ± 0.58a	8.50 ± 1.50b	15.13 ± 1.39c	16.18 ± 1.73c
Rha	18.78 ± 0.97b	33.37 ± 5.21de	29.11 ± 10.08cd	8.87 ± 4.95a	22.14 ± 5.56bc	39.15 ± 0.46ef	45.01 ± 2.82f
Fuc	1.26 ± 0.14 ab	2.56 ± 0.26c	2.18 ± 0.78abc	1.13 ± 0.81a	2.33 ± 1.00bc	2.51 ± 0.24c	2.87 ± 0.34c
Xyl	5.91 ± 1.52b	2.91 ± 1.20 ab	10.03 ± 1.86c	3.29 ± 2.58 ab	1.96 ± 0.39a	12.02 ± 0.90c	11.77 ± 2.51c
Glc	21.13 ± 3.42bc	19.83 ± 8.59bc	18.26 ± 7.22bc	6.27 ± 2.76a	12.83 ± 1.75 ab	16.06 ± 1.32bc	23.18 ± 1.70c
Man	204.20 ± 15.02b	178.16 ± 36.73b	172.17 ± 13.02b	127.02 ± 13.94a	174.64 ± 3.37b	171.10 ± 8.63b	183.31 ± 1.78b
TMSG	381.89 ± 17.76b	351.97 ± 16.52 ab	532.51 ± 20.72c	363.94 ± 40.76b	300.83 ± 34.51a	652.31 ± 22.16d	681.94 ± 47.88d
TMS	607.21 ± 0.69c	549.96 ± 13.84b	714.03 ± 10.29d	497.23 ± 55.44 ab	488.31 ± 39.13a	839.73 ± 19.60e	888.43 ± 44.41e
MP	255.25 ± 18.77b	222.70 ± 45.92b	215.22 ± 16.27b	158.77 ± 17.43 ab	218.31 ± 4.21b	214.21 ± 10.77b	229.14 ± 2.22b
PRAG	370.05 ± 31.96b	228.20 ± 12.50a	452.44 ± 51.93c	347.27 ± 8.31b	215.38 ± 10.10a	542.62 ± 28.80d	532.22 ± 31.04d
RG-II	148.15 ± 29.49a	343.82 ± 33.73cd	286.91 ± 25.78b	336.55 ± 10.50bcd	331.86 ± 25.70bc	384.23 ± 27.03d	362.54 ± 30.84cd
PRAG + RG-II	518.21 ± 31.04a	572.02 ± 21.59a	739.36 ± 36.78b	683.82 ± 7.75b	547.24 ± 35.73a	926.85 ± 1.77c	894.76 ± 61.89c
TSP	773.46 ± 26.38 ab	794.72 ± 61.12 ab	954.58 ± 20.52c	842.59 ± 19.17b	765.55 ± 39.93a	1141.06 ± 12.54d	1123.89 ± 59.67d

2-*O*MeFuc: 2-*O*-CH_3-fucose, 2-*O*MeXyl: 2-*O*-CH_3-xylose, Api: apiose, Kdo: 2-keto-3-deoxyoctonate ammonium salt, Ara: arabinose, Gal: galactose, GalA: galacturonic acid, GluA: glucuronic acid, Rha: rhamnose, Fuc: fucose, Xyl: xylose, Glc: glucose, Man: mannose, TMSG: total monosaccharides except mannose and glucose, TMS: total monosaccharides, MP: mannoproteins, PRAG: polysaccharides rich in arabinose and galactose, RG-II: rhamnogalacturonans type II, TSP: total soluble polysaccharides, W12: wine made from 12° Baumé grapes, WC: control wine, US: ultrasound application, E: enzyme addition, 3d: 3 days of maceration, 7d: 7 days of maceration. Different letters in the same row mean statistically significant differences ($p < 0.05$) (n = 3).

Table 2
Monosaccharide composition (mg/L) of polysaccharides and polysaccharides families (mg/L) in wines from 14° Baumé grapes.

Compound (mg/L)	Samples					
	W14–C3d	W14–C + E3d	W14-US3d	W14-US + E3d	W14–C7d	W14-US7d
2-*O*MeFuc	5.88 ± 0.92a	7.23 ± 0.70a	9.25 ± 0.54b	10.48 ± 0.59b	7.00 ± 0.35a	27.43 ± 1.46c
2-*O*MeXyl	3.27 ± 0.74b	1.46 ± 0.37a	4.88 ± 0.25cd	5.56 ± 0.92d	4.09 ± 0.09bc	14.15 ± 0.88e
Api	0.67 ± 0.24a	3.65 ± 1.16b	1.15 ± 0.14a	3.18 ± 0.37b	2.76 ± 0.41b	7.85 ± 0.49c
Kdo	2.08 ± 0.68a	2.18 ± 0.89a	2.74 ± 0.26a	1.91 ± 0.87a	2.43 ± 0.15a	6.80 ± 1.21b
Ara	70.74 ± 10.95b	38.29 ± 3.00a	90.60 ± 3.70b	32.76 ± 1.96a	121.30 ± 29.83c	241.41 ± 17.64d
Gal	333.35 ± 19.78b	360.16 ± 29.55bc	426.54 ± 25.63c	193.35 ± 1.38a	390.56 ± 8.73bc	1069.85 ± 75.64d
GalA	59.58 ± 14.69a	87.54 ± 14.90 ab	89.18 ± 12.83 ab	51.84 ± 24.28a	125.62 ± 33.25b	281.23 ± 25.41c
GluA	11.88 ± 2.39 ab	11.35 ± 0.60 ab	15.61 ± 1.24bc	7.44 ± 2.89a	21.02 ± 6.05c	44.02 ± 2.97d
Rha	26.53 ± 5.16a	42.09 ± 7.08bc	39.63 ± 2.88abc	27.59 ± 5.60 ab	54.12 ± 14.95c	115.50 ± 5.53d
Fuc	1.83 ± 0.46a	3.67 ± 0.30bc	2.88 ± 0.19b	2.67 ± 0.19 ab	3.99 ± 1.03c	9.02 ± 0.65d
Xyl	3.48 ± 1.47a	7.14 ± 0.80b	4.53 ± 0.17 ab	1.55 ± 0.57a	4.38 ± 0.07 ab	29.14 ± 4.19c
Glc	17.58 ± 6.96a	21.33 ± 3.85a	18.57 ± 2.18a	7.95 ± 3.01a	37.86 ± 16.71b	57.71 ± 10.11c
Man	199.55 ± 12.53a	194.09 ± 1.95a	192.22 ± 9.50a	193.90 ± 6.72a	205.88 ± 5.21a	628.75 ± 46.15b
TMSG	519.32 ± 51.33b	564.76 ± 42.00bc	686.99 ± 37.36cd	338.30 ± 35.36a	737.26 ± 76.14d	1846.40 ± 130.90e
TMS	736.44 ± 64.06b	780.20 ± 42.78b	897.78 ± 44.65bc	540.15 ± 34.55a	980.99 ± 89.51c	2532.85 ± 186.33d
MP	249.43 ± 15.66a	242.61 ± 2.44a	240.27 ± 11.88a	242.37 ± 8.39a	257.34 ± 6.51a	785.93 ± 57.68b
PRAG	483.61 ± 33.32b	488.34 ± 38.72b	610.83 ± 36.37c	236.51 ± 4.17a	603.82 ± 19.64c	1525.86 ± 110.67d
RG-II	240.65 ± 41.39a	254.88 ± 20.08a	374.20 ± 20.84b	424.26 ± 31.93b	289.37 ± 12.41a	1103.93 ± 60.43c
PRAG + RG-II	724.26 ± 62.84a	743.22 ± 44.01a	985.03 ± 49.04b	660.76 ± 27.77a	893.19 ± 7.24b	2629.824 ± 169.53c
TSP	973.69 ± 70.03 ab	985.84 ± 45.72 ab	1225.30 ± 60.64c	903.13 ± 27.79a	1150.53 ± 5.71bc	3415.74 ± 224.71d

2-*O*MeFuc: 2-*O*-CH_3-fucose, 2-*O*MeXyl: 2-*O*-CH_3-xylose, Api: apiose, Kdo: 2-keto-3-deoxyoctonate ammonium salt, Ara: arabinose, Gal: galactose, GalA: galacturonic acid, GluA: glucuronic acid, Rha: rhamnose, Fuc: fucose, Xyl: xylose, Glc: glucose, Man: mannose, TMSG: total monosaccharides except mannose and glucose, TMS: total monosaccharides, MP: mannoproteins, PRAG: polysaccharides rich in arabinose and galactose, RG-II: rhamnogalacturonans type II, TSP: total soluble polysaccharides, W12: wine made from 14° Baumé grapes, WC: control wine, US: ultrasound application, E: enzyme addition, 3d: 3 days of maceration, 7d: 7 days of maceration. Different letters in the same row mean statistically significant differences ($p < 0.05$) (n = 3).

wines, could be an oenological technique to modulate the sensation of astringency in wines made from immature grapes. Red wines made from unripe grapes are more tannic and astringent than those from ripe grapes. The polysaccharide composition of sonicated wines showed a PRAG content higher than RG-II, the same profile as that observed in the control wines, and different from that obtained in the wines made with the addition of enzymes (Table 1). Sonication maintained the composition of the polysaccharides released from the grape cell walls in the wines, increasing the content of both the PRAG and RG-II families. The polysaccharides of the grape cell walls and those released during the maceration-fermentation and pressing of the marc (Martínez-Lapuente, Guadalupe, Pérez-Porras, et al., 2021) have the capacity to interact with the tannins (Bautista-Ortín, Cano-Lechuga, Ruiz-García, & Gómez-Plaza, 2014; Bindon, Smith, Holt, & Kennedy, 2010; Osete-Alcaraz et al., 2020). Probably, the chains of arabinans, galactans, galacturonans, arabinogalactans, and arabinogalactan proteins from the grape cell wall matrix and those released by the action of added enzymes/ethanol or by the action of US/ethanol interacted with the tannins solubilized in the must during the maceration-fermentation and pressing of the marc reducing the PRAG content of the wines W12–C + E3d and W12-US3d. However, the reduction of the PRAG content in sonicated wines was not observed, probably due to a greater release caused by US. These results suggest that US treatment was more effective in releasing PRAG. Osete-Alcaraz et al. (2020) observed that the presence of tannins in a model solution containing the cell wall skin of Monastrell skin resulted in a decrease in the content of higher degree of polymerization polysaccharides released when pectin-lyase plus polygalacturonase were present, while low molecular weight polysaccharides showed a reduced ability to interact with the added tannins. Ayestarán et al. (2004) and Diez, Guadalupe, Ayestarán, and Ruiz-Larrea (2010) analyzed the composition of polysaccharides with a weight <6000 Da from Tempranillo wine and it was formed by a mixture of oligosaccharides or fragments of PRAG, MP, RG-II monomers, and HL.

The added E and US did not degrade the RG-II molecule, probably due to the complexity of its structure. RG-II is an ubiquitous molecule with an extremely conserved and identical structure in the grape cell walls and in the wine (Canalejo et al., 2021). However, the E were more effective in the release of RG-II than the US. Probably the rigidity of the cell walls of the immature grape limited the effect of US as observed in the 14° Baumé grape (Martínez-Lapuente, Guadalupe, Ayestarán, et al., 2021). Guadalupe and Ayestarán (2007) observed that RG-II needed more maceration time to solubilize, as it was more tightly bound to the cell wall matrix of grape cell walls, compared to the rapid solubilization of the PRAG that began from the beginning of the maceration. This different behavior could explain the preferential interaction of solubilized tannins with PRAG released by the action of E and US.

When comparing the content of RG-II + PRAG of the wines obtained with the combined treatments E + US and US + E with the wines made with each technique separately, it was observed that the combined treatment, regardless of the order of addition of the E, did not improve the release of grape polysaccharides into the wine (Table 1). However, the order of E addition affected the polysaccharide composition profile of the wines. The polysaccharide composition of the wines with the E addition just before US (E + US) was similar to those wines only treated with E (RG-II > PRAG). The wines with the E addition after US (US + E) showed a similar composition to the application of US separately (PRAG > RG-II) (Table 1). The results also indicated that the application of US alone obtained a similar content of PRAG + RG-II than the combined treatment US + E, and that the use of E alone was also sufficient to obtain a PRAG + RG-II content similar to that obtained in the E + US treatment. However, the PRAG + RG-II content of the wines with the addition of E after US was significantly higher than obtained in the E + US wines (Table 1). Contradictory results on the effect of US on enzyme activity have been found in literature. Some authors showed that US may inactivate enzymes (Vercet, Burgos, Crelier, & López-Buesa, 2001; Zhang et al., 2017) while others point out that it improves their activity (Chen et al., 2014; Yachmenev, Condon, Klasson, & Lambert, 2009; Nadar, Rao, & Rathod, 2018). Our results could not confirm the inactivation of E by cavitational effect. Pérez-Porras et al. (2022) studied the effect of the application of a combination of both techniques on the chromatic characteristics and tannins in wines in an identical experiment and only observed a synergistic effect, compared with the use of one or the other technique, when the E were applied after US. Several studies (Castro-Lopez, Gómez-Plaza, Ortega-Regules, Lozada, & Bautista-Ortin, 2016; Osete-Alcaraz et al., 2019; Osete-Alcaraz et al., 2020, Pérez-Porras et 2021; Ruiz-García, Smith, & Bindon, 2014)

observed that the degradation or elimination of cell wall pectic polysaccharides by E or by US or a combination of both techniques (US + E) promoted a higher content of soluble tannins, reducing the adsorption of tannins by the grape cell walls. However, the favorable effect on tannins caused by these techniques may not mean a higher content of cell wall polysaccharides in the wines.

The PRAG + RG-II content of wines with a maceration time of 7 days was similar (Table 1). However, the wines W12-US3d and those of combined treatment W12-US + E3d showed the PRAG + RG-II values closest to those of seven-day maceration wines (W12–C7d and W12-US7d) (Table 1). The wines with long maceration showed a similar content of PRAG, and reached the highest values compared to the other wines (Table 1). The RG-II contents of wines with long maceration and wines with short maceration (W12–C + E3d and W12-US + E3d) was similar, and that of wines W12-US3d, W12-E + US3d and W12-US + E3d was also similar between them. Therefore, wines with a maceration time of three days and E or US + E showed the same content of RG-II to those of maceration of seven days.

In general, the content of mannose, glucose and MP was similar among the wines (Table 1). These results indicated that the application of the techniques separately or the combination of both techniques did not degrade the cell walls of the yeasts.

As regards the results obtained with the most ripen grapes, wines with higher alcohol content had higher TMS, TMSG and TSP content than wines with lower alcohol content (Tables 1 and 2). A higher degree of maturation implies a higher degree of degradation of the grape cell walls and a higher ethanol content in the wine, factors that favored the release of polysaccharides from the grape cell walls and yeasts into the wines.

Considering the lower content of PRAG + RG-II, the same experiment (E + S) was not repeated with the ripest grapes. The E addition to the ripest grape, with a higher degree of cell wall degradation due to ripening (Gao et al., 2015; Gao, Fangel, Willats, Vivier, & Moore, 2021), implied a content of galactose, TMSG, PRAG and RG-II similar to observed in W14–C3d wines (Table 2). Results indicated a low effect of enzymes when these are used during the vinification of grapes with an advanced degree of ripening, possibly due to the greater degradation occurring naturally in grape structures with ripening (Bautista-Ortín, Fernández-Fernández, López-Roca, & Gómez-Plaza, 2007). On the other hand, cavitation caused a higher degree of release of TMSG, 2-*O*MeFuc, 2-*O*MeXyl, RG-II and PRAG from the cell wall compared to wines W14–C + E3d and W14–C3d. The polysaccharide profile of the wines was similar, with a PRAG content higher than that of RG-II, result that differs from the polysaccharide composition of the wines made with the addition of E to less ripe grape. Sonication treatment was more effective in releasing PRAG + RG-II than addition of enzymes to ripe grapes. The application of the sonication technique separately and the combined US + E technique was equally effective in immature grapes (Tables 1 and 2).

The combined treatment US + E did not imply an improvement in the wines with higher alcohol content of TMSG, TMS and RG-II + PRAG compared to the wines obtained with the techniques applied separately (Table 2), although the combined treatment modified the polysaccharide profile of the wines (RG-II > PRAG) (Table 2). Regardless of the level of grape ripening, our results indicated that the combined US + E treatment did not have a synergistic effect on the release of polysaccharides from the grapes into the wines.

The maceration time of 7 days in the most mature sonicated grapes increased very significantly the concentration of RG-II and PRAG in wines, reaching the highest values of RG-II, PRAG and PRAG + RG-II of all the wines (Table 2). These results differed from those obtained with less mature grapes, and indicated that, for the same maceration time, sonication was more effective when it is applied to more mature grapes. However, the PRAG + RG-II content of wines only sonicated with three days of maceration was similar to control wines with maceration time of 7 days. These results agree with those observed by Martínez-Lapuente, Guadalupe, Ayestarán, et al. (2021), which indicated that sonication could be a useful technology to increase the content of polysaccharides from grapes, allowing a reduction of the maceration time.

MP content was similar in wines with higher alcohol content, except for long-maceration sonicated wines (Table 2). The yeast strain was the same, it was added after sonication in all the tests, and the fermentation kinetics was similar to the rest of the fermentations (data not shown). Therefore, the higher content of MP observed in W14-US7d wine could be due to part of the mannose coming from the grape cell wall mannans released by the longer sonication time used.

3.2. Principal factors of variability of the content of wine monosaccharides and polysaccharides families

A multivariate analysis of variance (MANOVA) was conducted in wine samples obtained with short maceration to analyze the effect of grape ripening degree (RD), sonication (US) and addition of enzymes (E) on wine monosaccharides and polysaccharides (Table 3). According to Pillai trace and Wilks' lambda statistics, ripening degree, ultrasound application, enzyme addition, and their interactions (RDxUS, RDxE and USxE) were found to be significant for all monosaccharides and polysaccharides ($p < 0.05$). The more significant effect was observed in US treatment ($p < 0.01$) (Table 3).

It was observed in Table 3 that none of the considered factors or their interactions presented a dominant effect of variation for the concentration of monosaccharides and polysaccharides in the wines, as they did not reach values of % variance greater than 80%.

Except for 2-*O*-CH_3-xylose, and glucose, the degree of grape ripening had a significant effect ($p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$) on the average content of monosaccharides and polysaccharides, confirming the higher extraction in wines made from more mature grapes. The 14 °Baumé grapes had a greater effect on the glycosyl and polysaccharide families than the 12 °Baumé grapes.

Sonication caused a very significant higher content of 2-*O*-CH_3-fucose, 2-*O*-CH_3-xylose, arabinose, RG-II, PRAG + RG-II and TSP ($p \leq 0.001$ and $p \leq 0.01$) at favor of sonicated wines. Mannose, glucose and MP content was higher ($p \leq 0.01$) in control wines. The content of the rest of the monosaccharides, and PRAG were similar between the sonicated and control wines. The interaction between RD and US were significant for 2-*O*-CH_3-fucose, galactose, xylose, mannose, RG-II, PRAG, PRAG + RG-II and MP, which indicated that the effect of the grape ripening was modified to some extent by the cavitation phenomenon.

The addition of E positively affected the average content of 2-*O*-CH_3-fucose, apiose, RG-II ($p \leq 0.001$) and fucose ($p \leq 0.05$) in the wines. However, the non-addition of E (control wine) significantly increased ($p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$) the content of arabinose, galactose, xylose, glucuronic acid, glucose, mannose, PRAG, PRAG + RG-II, MP and TSP in control wines. The RDxE interaction decreased the percent variance with respect to RD and E, in arabinose, galactose, mannose, PRAG, PRAG + RG-II, MP and TSP, which were positively affected when more mature grapes were used. The USxE interaction increased the percent variance with respect to US and E, in the content of Kdo, glucuronic acid, rhamnose, fucose and xylose, and TSP with the E addition after US treatment.

4. Conclusions

This work presents for the first time the effect of the combined technique US + E on the content of the wine polysaccharide composition compared to wine made with the techniques applied separately, US or E.

The factors studied in wine samples obtained with short maceration, ripening degree, ultrasound application, enzyme addition, and their interactions (RDxUS, RDxE and USxE) were found to be significant for all monosaccharides and polysaccharides. Sonication of crushed grapes increased the PRAG + RG-II content of the wines. Sonication was more effective than enzymes in terms of increasing PRAG + RG-II content in wine. Enzyme addition to sonicated grapes did not have a synergistic

Table 3
Monosaccharide composition and polysaccharides families (mg/L) and percentage of variance attributable to ripening degree, sonication treatment and enzyme addition and the interaction of them at the time of bottling for wines made with 3 days of maceration.

Compounds	mg/L						% variance							
	RD		US		E									
	12 °Baumé	14 °Baumé	Control without US	US	Control without E	E	RD	US	E	RDxUS	RDxE	USxE	RDxUSxE	Residual
2-*O*MeFuc	6.23	8.21	6.29	8.15	6.30	8.15	23.03***	20.34***	20.01***	12.20***	1.83 NS	7.06**	6.28**	9.25
2-*O*MeXyl	4.70	3.79	2.88	5.61	3.84	4.65	3.96 NS	35.73**	3.16 NS	0.07 NS	9.12 NS	0.20 NS	5.14 NS	42.61
Api	0.99	2.16	1.68	1.48	1.00	2.15	23.76***	0.70 NS	22.85***	0.72 NS	31.86***	5.28*	0.11 NS	14.72
Kdo	1.11	2.23	1.74	1.60	1.72	1.62	38.02***	0.55 NS	0.33 NS	3.27 NS	2.06 NS	20.64**	3.93 NS	31.19
Ara	41.35	58.10	43.71	55.74	68.63	30.82	13.06***	6.75***	66.58***	1.11 NS	2.51**	5.17***	0.22 NS	4.62
Gal	254.95	328.35	281.59	301.71	330.44	252.85	21.03***	1.58 NS	23.49***	12.65***	2.56**	15.38***	17.63***	5.68
GluA	7.76	11.57	10.34	8.99	11.76	7.57	20.78***	2.59 NS	25.07***	2.23 NS	0.04 NS	27.36***	0.44 NS	21.49
Rha	22.53	33.96	30.19	26.30	28.51	27.98	26.20***	3.04 NS	0.06 NS	2.04 NS	1.06 NS	48.86***	0.66 NS	18.09
Fuc	1.78	2.76	2.33	2.21	2.04	2.51	30.66***	0.42 NS	7.11*	0.62 NS	3.76 NS	38.76***	0.18 NS	18.50
Xyl	5.53	4.17	4.86	4.85	5.99	3.72	5.81*	0.00 NS	16.13**	16.10**	21.33***	21.16***	1.64 NS	17.82
Glc	16.37	16.36	19.97	12.76	18.89	13.85	0.00 NS	26.60**	13.01*	0.52 NS	1.32 NS	20.14**	0.44 NS	37.97
Man	170.39	194.94	194.00	171.33	192.04	173.29	20.73**	17.68**	12.09*	12.30*	9.77*	0.31 NS	1.48 NS	25.63
RG-II	278.86	323.50	246.88	355.48	262.48	339.88	6.97***	41.23***	20.94***	6.42**	7.16***	2.65*	7.23***	7.40
PRAG	349.49	454.82	392.55	411.76	479.23	325.08	17.45***	0.58 NS	37.38***	10.45***	1.48*	11.52***	16.99***	4.15
PRAG + RG-II	628.35	778.32	639.43	767.24	741.72	664.96	31.17***	22.64***	8.17***	2.07*	7.98***	17.74***	4.74**	5.50
MP	212.99	243.67	242.50	214.16	240.04	216.62	20.74**	17.68**	12.08***	12.30*	9.77*	0.31 NS	1.48 NS	25.63
TSP	841.34	1021.99	881.93	981.40	981.76	881.57	42.09***	12.76***	12.94***	0.29 NS	3.88**	17.62***	3.26*	7.16

	RD	US	E	RDxUS	RDxE	USxE	RDxUSxE
Pillai's Trace	0.984	0.993	0.992	0.985	0.987	0.978	0.973
p	*	**	*	*	*	*	NS
Wilks Lambda	0.016	0.007	0.008	0.015	0.013	0.022	0.027
p	*	**	*	*	*	*	NS

2-*O*MeFuc: 2-*O*-CH3-fucose, 2-*O*MeXyl: 2-*O*-CH3-xylose, Api: apiose, Kdo: 2-keto-3-deoxyoctonate ammonium salt, Ara: arabinose, Gal: galactose, GluA: glucuronic acid, Rha: rhamnose, Fuc: fucose, Xyl: xylose, Glc: glucose, Man: mannose, RG-II: rhamnogalacturonans type II, PRAG: polysaccharides rich in arabinose and galactose, MP: mannoproteins, TSP: total soluble polysaccharides, RD: ripening degree, 12: wine made from 12° Baumé grapes, 14: wine made from 14° Baumé grapes, C: control wine, US: ultrasound application, E: enzyme addition. Statistically significant at $*p \leq 0.05$; $**p \leq 0.01$ and $***p \leq 0.001$, respectively. NS, not significant.

effect. In fact, the PRAG + RG-II content of the W12-US + E3d wines was like the W12-US3d wines, and to wines made from mature grapes (WT14-US + E3d). However, the PRAG + RG-II content of the W12-US3d and W12-US + E3d wines was the closest to the control wines and those vinified with US with 7 days of maceration, or even the W14-US3d wines exceeded the control wine W14–C7d. However, lower PRAG + RG-II content was observed in W12-E + US3d wine. Nevertheless, future studies with other conditions of frequency, power and power density of ultrasound could improve the enzymatic activities and could reach a possible synergistic effect. Sonication could be a suitable technology to increase the content of polysaccharides from grapes, allowing a reduction of the maceration time. Future studies will evaluate the effect of sonication treatment on modulating the intensity of astringency, necessary in wines made from unripe grapes.

Another interesting result was that sonication treatment maintained the same profile of total composition of polysaccharides than the control wine (PRAG > RG-II). On the contrary, the E and E + US treatments applied in immature grapes and US + E applied in ripe grapes modified the profile of total composition of polysaccharides (RG-II > PRAG), and thus the physicochemical properties of the PRAG were minor.

The treatments studied did not affect the MP content of the yeasts in the wines with a similar alcohol content (except in the W14-US7d wines). The higher alcoholic degree favored the release of MP.

CRediT authorship contribution statement

Leticia Martínez-Lapuente: Conceptualization, Investigation, Formal analysis, Methodology, Writing – original draft, Were in charge of the conceptualization as well as of the investigation, Did the analysis of the data and were in charge of the methodology, oversaw the writing of the original draft, Reviewing, and editing of the final manuscript. **Zenaida Guadalupe:** Writing – review & editing, Writing – original draft, Writing, reviewing, and editing of the final manuscript. **Belén Ayestarán:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Were in charge of the conceptualization as well as of the investigation, Oversaw the writing of the original draft, Data curation, and The writing, reviewing, and editing of the final manuscript. **Paula Pérez-Porras:** Conceptualization, Investigation, Formal analysis, Methodology, Charge of the conceptualization as well as of the investigation. **Ana Belén Bautista-Ortín:** Conceptualization, Investigation, Formal analysis, Methodology, Reviewing, and editing of the final manuscript, Charge of the conceptualization as well as of the investigation. **Encarna Gómez-Plaza:** Conceptualization, Investigation, Formal analysis, Methodology, Reviewing, and editing of the final manuscript, Charge of the conceptualization as well as of the investigation.

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.

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