

## Polysaccharides and Oligosaccharides Produced on Malvar Wines Elaborated with *Torulaspora delbrueckii* CLI 918 and *Saccharomyces cerevisiae* CLI 889 Native Yeasts from D.O. "Vinos de Madrid"

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**Polysaccharides and Oligosaccharides Produced on Malvar Wines Elaborated with *Torulaspota delbrueckii* CLI 918 and *Saccharomyces cerevisiae* CLI 889 Native Yeasts from D.O. “Vinos de Madrid”**

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**1 ABSTRACT**

2 Polysaccharides and oligosaccharides released into Malvar white wines  
3 elaborated through pure, mixed and sequential cultures with *Torulasporea*  
4 *delbrueckii* CLI 918 and *Saccharomyces cerevisiae* CLI 889 native yeasts from  
5 D.O. "Vinos de Madrid" were studied. Both fractions from different white wines  
6 were separated by high-resolution size-exclusion chromatography. Glycosyl  
7 composition and linkages of wine polysaccharides were determined by GC-EI-  
8 MS chromatography. Molar-mass distributions were determined by SEC-  
9 MALLS and intrinsic viscosity by differential viscometer. Yeast species and type  
10 of inoculation have a significant impact on wine carbohydrate composition and  
11 structure. Mannose residues from mannoproteins were significantly predominant  
12 in those cultures where *T. delbrueckii* was present in the fermentation process in  
13 comparison with pure culture of *S. cerevisiae*, whereas galactose residues from  
14 Polysaccharides Rich in Arabinose and Galactose presented higher values in pure  
15 culture of *S. cerevisiae*, indicating that *S. cerevisiae* released less mannoproteins  
16 than *T. delbrueckii*. Moreover, we reported structural differences between  
17 mannoproteins released by *T. delbrueckii* CLI 918 and those released by *S.*  
18 *cerevisiae* CLI 889. These findings help to provide important information about  
19 the polysaccharides and oligosaccharides released from cell wall of Malvar  
20 grapes and the carbohydrates released from each yeast species.

21

22 **Keywords:** Malvar white wines, native yeast, mannoproteins, polysaccharides,  
23 oligosaccharides

## 24 INTRODUCTION

25 The study of wine polysaccharides has acquired great interest in recent years  
26 owing to its role on a number of technological and sensorial properties in wine.  
27 Numerous studies have clearly proven that these macromolecules have many  
28 positive oenological features such as protection against tartrate salt  
29 crystallization;<sup>1</sup> prevention of protein haze formation in white wines;<sup>2</sup> reduction  
30 of astringency;<sup>3,4</sup> interaction with tannins;<sup>5</sup> increased sweetness and increased  
31 body and mouth feel;<sup>3</sup> formation of specific coordination complexes with Pb<sup>+2</sup>  
32 ions.<sup>6</sup> In sparkling wines, foam characteristics have been correlated with the type,  
33 the molecular weight and the glycosyl composition of polysaccharides.<sup>7</sup>  
34 Polysaccharides found in wine originate from grapes, yeasts and bacteria during  
35 the winemaking. Those originating from grape cell walls are Polysaccharides  
36 Rich in Arabinose and Galactose [PRAGs, which comprise arabinans,  
37 arabinogalactans, and arabinogalactan proteins],<sup>8</sup> homogalacturonans<sup>9</sup> and  
38 rhamnogalacturonans of type II (RG-II)<sup>10</sup> whereas those released by  
39 microorganisms are mainly mannoproteins (MPs) produced by yeasts during the  
40 alcoholic fermentation or ageing on lees,<sup>11</sup> and glucan-like structures by  
41 bacteria.<sup>12</sup>  
42 However, the oligosaccharides composition in wine have only recently been  
43 studied.<sup>13-16</sup> These molecules are linked to defensive responses of plants.<sup>17</sup>  
44 Moreover, oligosaccharides can be found in important medicinal and food  
45 applications.<sup>18</sup> According to their influence on the wine, it should be noted their  
46 ability to chelate cations which can be important for wines. The structure and the

47 amounts of oligosaccharides, as well as in the case of polysaccharides, depend on  
48 several factors, such as the grape origin,<sup>19</sup> the grape cultivar<sup>16</sup> and the  
49 winemaking process and can be modified by enzyme treatment.<sup>19</sup> Therefore, it  
50 would appear to be necessary to go further with the identification, quantification  
51 and composition of oligosaccharides in different wines in order to better  
52 understand their influence on technological and organoleptic properties.

53 Recently, Giovani et al.<sup>20</sup> have shown the high capacity of non-*Saccharomyces*  
54 wine yeasts to release important polysaccharides into wine during alcoholic  
55 fermentation. The natural capacity of non-*Saccharomyces* yeasts to release  
56 complex carbohydrates could be consider as selection criteria of these yeasts to  
57 wine elaboration.<sup>21</sup> The role of non-*Saccharomyces* yeasts in winemaking has  
58 been revised in recent years, the use of controlled mixed fermentations as a  
59 biotechnological tool has been promoted in order to enhance special and specific  
60 characteristics of a wine thus improve their complexity.<sup>21</sup> This practice has also  
61 been reported as being able to increase some desirable metabolites, such as some  
62 acetate esters<sup>22</sup> and glycerol.<sup>21</sup> Moreover, some non-*Saccharomyces* yeasts have  
63 been reported as being able to release more polysaccharides than *S. cerevisiae*  
64 strains during alcoholic fermentation.<sup>20</sup>

65 *T. delbrueckii* was one of the first commercially available non-*Saccharomyces*  
66 for the winemaking industry. During wine fermentation, *T. delbrueckii* in pure  
67 cultures and mixed cultures with *S. cerevisiae* has produced wines with higher  
68 sensory complexity and floral and fruity aromas because of its capacity of  
69 produce high concentration of higher alcohols, esters, terpenes and phenolic

70 aldehydes as well as other compounds like 2-phenylethanol and linalool.<sup>23</sup> It has  
71 also been described its ability to produce wines with lower volatile acidity,  
72 acetaldehyde and acetoin.<sup>24</sup> Depending on the strain, *T. delbrueckii* can produce  
73 low/medium glycerol, succinic acid and polysaccharides.<sup>20,25,26</sup> Moreover,  
74 *Torulaspota* genera is reported good producer of enzymes such as  $\beta$ -  
75 glucosidases, pectinases, proteases and those related to xylan degradation but this  
76 capacity of enzymes secretion also depends on the yeast strain analyzed.<sup>27</sup>

77 The Denomination of Origin (D.O.) “Vinos de Madrid”, created in 1990, is  
78 located in the centre of Spain (between 40° 16’ N and 40° 24’ N latitude) and  
79 covers an area of 8.390 ha. The climate of this agronomy zone is continental with  
80 temperatures ranging from -8 °C minimum in winter to 41 °C maximum in  
81 summer,<sup>28</sup> and the annual rainfall means are between 460 and 660 mm. The most  
82 cultivated grape cultivars are Airén and Malvar (white), and Garnacha and  
83 Tempranillo (red) (all of them *Vitis vinifera* L. cv.). Malvar is an autochthonous  
84 cultivar for this D.O., while Airén, Garnacha and Tempranillo have major  
85 extensions all over the Iberian Peninsula. Part of the economic development of  
86 this area is based on wine production with winemakers searching for the  
87 production of high quality wines with singular identity. The selection of native  
88 yeasts is an emerging tool which contributes to elaborate new styles of wines  
89 more competitive in the market.<sup>29</sup>

90 The aim of this study was to determine the polysaccharides and oligosaccharides  
91 release into Malvar white wines elaborated with *T. delbrueckii* CLI 918 and *S.*  
92 *cerevisiae* CLI 889 native yeasts from D.O. “Vinos de Madrid” in pure, mixed

93 and sequential cultures made with both strains. To date, this is the first report of  
94 the polysaccharide and oligosaccharide composition in regional Malvar wines  
95 using autochthonous *S. cerevisiae* and non-*Saccharomyces* yeasts from this  
96 region.

## 97 **MATERIALS AND METHODS**

### 98 **Chemicals**

99 Analytical Reagent grade chemicals were used in the present study. Ammonium  
100 sulphate supplied by Panreac Quimica (Barcelona, Spain) and Enozym Altair  
101 pectolytic enzymes obtained from Agrovin (Ciudad Real, Spain), were used  
102 during must preparation. The following compounds were used by  
103 polysaccharides and oligosaccharides isolation and analysis in the studied wines.  
104 Sodium chloride, phosphorus pentoxide, hydrogen chloride, trifluoroacetic acid,  
105 ammonia, acetone, glacial acetic acid, ethyl acetate, acetic anhydride, perchloric  
106 acid 70%, 1-methylimidazole, chloroform, and n-hexane were obtained from  
107 Merck (Darmstadt, Germany). Methanol anhydrous, sodium borodeuteride and  
108 myo-inositol were purchased from Sigma-Aldrich (St Louis, MO, USA).  
109 Polyamide SC6 was supplied by Macherey-Nagel (Düren, Germany).  
110 Ammonium formiate was supplied by Acros Organics (Geel, Belgium). Tri-Sil  
111 HTP Reagent was obtained from Thermo Scientific (Waltham, MA, USA).

### 112 **Yeast strains**

113 The yeast strains used for the elaboration of the wines were the *Saccharomyces*  
114 CLI 889 strain, selected as native strain from D.O. “Vinos de Madrid” and  
115 characterized in the IMIDRA’s laboratories based on some established and

116 desirable oenological criteria<sup>30</sup>. This strain has been deposited in Spanish Type  
117 Culture Collection (CECT 13145). And *Torulaspota delbrueckii* CLI 918,  
118 selected according to its biotechnological potential.<sup>28</sup> *T. delbrueckii* CLI 918 has  
119 been described earlier as a strain with potential interest for its contribution to the  
120 aromatic wine profile adding flowery and fruity notes and its use was considered  
121 interesting in mixed starter cultures with *S. cerevisiae*,<sup>28</sup> and this strain has also  
122 shown good fermentative capacity under different stress conditions.<sup>31</sup>

### 123 **Vinification procedure**

124 Grapes from Malvar cultivar (*Vitis vinifera* L. cv.) were hand-collected from  
125 IMIDRA's experimental vineyard located in Madrid winegrowing region, Spain  
126 (40° 31' N Longitude, 3° 17' W Latitude, and 610 m Altitude) during the 2010  
127 vintage at commercial maturity (21.5 °Brix, equivalent to about 205 g L<sup>-1</sup> of  
128 sugars). The grapes were gently destemmed and pressed and, the must was  
129 racked, homogenized and clarified by pectolytic enzymes (1 g/hL) at 4 °C. Clear  
130 must was supplemented with nitrogen by adding ammonium sulphate (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>  
131 up to a level of 250 mgN L<sup>-1</sup> before beginning the alcoholic fermentation.

132 Triplicate experiments were carried out in sterile flasks with 1 L of pasteurized  
133 (as indicated in García et al., 2017<sup>32</sup>) Malvar must with constant agitation (150  
134 rpm) in an 18 °C temperature controlled room. Fermentations were divided into  
135 pure, mixed and sequential cultures. Pure cultures were independently inoculated  
136 with 10<sup>6</sup> cells mL<sup>-1</sup> CLI 889 *S. cerevisiae* strain (culture considered as control)  
137 and 10<sup>6</sup> cells mL<sup>-1</sup> of CLI 918 *T. delbrueckii* strain. Mixed fermentation trials  
138 were simultaneously inoculated with 10<sup>6</sup> cells mL<sup>-1</sup> of *T. delbrueckii* and 10<sup>6</sup>

139 cells mL<sup>-1</sup> of *S. cerevisiae* strain. Sequential fermentation trials, were inoculated  
140 with 10<sup>6</sup> cells mL<sup>-1</sup> of the *T. delbrueckii* culture at first and the addition of the *S.*  
141 *cerevisiae* strain (10<sup>6</sup> cells mL<sup>-1</sup>) took place when the wine contained 5 % alcohol  
142 (v/v). The fermentation progress was monitored by automatic weight using the  
143 software OPCEX3 (Resolvica Inc., Chanhassen, MN) every 24 h until the end of  
144 the fermentation (constant weight). After fermentation, yeast cells and wines  
145 were separated by centrifugation (9000 rpm, 10 min, 4 °C); the pellets were  
146 discarded and the wines were kept frozen until analysis.

147 Principal oenological parameters of final wines were measured by Fourier  
148 transform infrared spectroscopy in the laboratories of Liec Agroalimentaria S. L.  
149 (Manzanares, Spain), an accredited laboratory for physico-chemical analysis in  
150 wines to conform to UNE-EN ISO/IEC 17025:2005 rules.

### 151 **Isolation of polysaccharide and oligosaccharide fractions**

152 The polysaccharide and oligosaccharide fractions were isolated as previously  
153 described.<sup>4,7,13,15</sup> Briefly, Malvar white wines (5 mL) were partially depigmented  
154 in polyamide CC6 columns, particle size 0.05–0.16 previously equilibrated with  
155 1 M NaCl. Wine polysaccharides and oligosaccharides passed through the  
156 column and were eluted with two bed volumes of 1 M NaCl. The eluted fraction  
157 was concentrated in a centrifugal evaporator (EZ-2, Genevac, Ipswich, UK).  
158 High-resolution size-exclusion chromatography (HRSEC) was performed by  
159 loading concentrated total wine carbohydrate on a system composed by a  
160 rheodyne sampling injector with a loop of 2 mL, an Intelligent pump 301  
161 (FLOM, France), and a fraction collector Frac-920 (GE Healthcare Bio-Sciences,

162 Pittsburgh, USA). Elution was performed on a Superdex-30 HR column (60 x 1.6  
163 cm, Pharmacia, Sweden) with a precolumn (0.6 x 4 cm) equilibrated at 1 mL  
164 min<sup>-1</sup> with 30 mM ammonium formate pH 5.6. The elution of polysaccharides  
165 and oligosaccharides was monitored with a RI 101 (Shodex Showa Denko,  
166 Japan) refractive index combined with Chromeleon software (Dionex,  
167 Sunnyvale, CA). The polysaccharide fraction was eluted between 41 and 55 min,  
168 while oligosaccharide fraction was collected between 56 and 93 min.<sup>4,7,16</sup> The  
169 isolated fractions were freeze-dried, redissolved in water, and freeze-dried again  
170 four times to remove the ammonium salt.

### 171 **Glycosyl-Linkage determination**

172 The glycosyl-linkage compositions were determined by GC-MS of the partially  
173 methylated alditol acetates. One milligram of polysaccharides in 0.5 mL of  
174 dimethyl sulfoxide was methylated using methyl sulfinyl carbanion and methyl  
175 iodide.<sup>33</sup> Methylated samples were hydrolyzed with 2 M of trifluoroacetic acid  
176 for 75 min at 120 °C. The released methylated monosaccharides were converted  
177 in their corresponding alditols by treatment with NaBD<sub>4</sub> and then acetylated by  
178 adding ethyl acetate, acetic anhydride and perchloric acid.<sup>34</sup> Partially methylated  
179 alditol acetates were analyzed by GC-EI-MS using a DB-1 capillary column (30  
180 m x 0.25 mm i.d., 0.25 μm film), temperature programming 135 °C for 10 min  
181 and then 1.2 °C min<sup>-1</sup> to 180 °C, with hydrogen as the carrier gas on a Shimadzu  
182 GCMS-QP2010SE gas chromatograph (Shimadzu, Kyoto, Japan).<sup>35</sup> Myo-inositol  
183 was used as internal standard and, to verify the rate of methylation. Response  
184 factors of partially methylated alditol acetates are those described by Sweet et

185 al.<sup>36</sup> The validity of the used methods and their repeatability were checked  
186 according to Vidal et al.<sup>35</sup>

### 187 **Oligosaccharides analysis**

188 The neutral and acidic sugar compositions were determined after solvolysis with  
189 anhydrous MeOH containing 0.5 M HCl (80 °C, 16h), by GC of their per-O-  
190 trimethylsilylated methyl glycoside derivatives.<sup>37</sup> The TMS derivatives were  
191 separated on two DB-1 capillary columns (30 m x 0.25 mm i.d., 0.25 µm film)  
192 (temperature programming 120–145 °C at 1.5 °C min<sup>-1</sup>, 145–180 °C at 0.9 °C  
193 min<sup>-1</sup>, and 180–230 °C at 50 °C min<sup>-1</sup>), coupled to a single injector inlet through  
194 a two-holed ferrule, with hydrogen as the carrier gas on a Shimadzu GCMS-  
195 QP2010SE gas chromatograph. The outlet of the one column was directly  
196 connected to a FID at 250 °C, and the second column via a deactivated fused-  
197 silica column (0.25 m x 0.11 µm i.d.) was connected to a mass detector. Samples  
198 were injected in the pulse split mode with a split ratio of 20:1. The transfer line to  
199 the mass was set at 280 °C. Electron ionization mass spectra were obtained from  
200 m/z 50 to 400 every 0.2 s in the total ion-monitoring mode using an ion source  
201 temperature of 200 °C, a filament emission current of 60 µA, and an ionization  
202 voltage of 70 eV.

### 203 **Determination of molar mass of wine polysaccharides**

204 Molar mass distributions, molar weight and number-average mass ( $M_w$  and  $M_n$  in  
205 g/mol), polydispersity index ( $M_w/M_n$ ), and intrinsic viscosity ( $[\eta]$  in mL g<sup>-1</sup>) were  
206 determined at 25 °C by coupling size exclusion chromatography with a  
207 multiangle light scattering device (MALLS), a differential viscometer, and a

208 differential refractive index detector. SEC elution was performed on an OH-pack  
209 guard followed by two serial Shodex OH-pack KB-804 and KB805 columns (0.8  
210 x 30 cm; Shodex Showa Denko, Japan) at a 1 mL min<sup>-1</sup> flow rate in 0.1 M LiNO<sub>3</sub>  
211 after filtration through a 0.1 μm filter unit. The MALLS photometer, a DAWN-  
212 HELEOS from Wyatt Technology Inc. (Wyatt Technology Corporation, Santa  
213 Barbara, CA, USA), was equipped with a GA-AS laser (λ = 658 nm). The  
214 differential viscometer detector (Viscostar II, Wyatt Technology Inc., USA) was  
215 equipped with a 4-capillary bridge design. The concentration of each eluted  
216 polysaccharide was determined using the differential refractive index detector  
217 (Optilab TrEX, Wyatt Technology Inc., USA). All collected data were analyzed  
218 using Astra V 6.0.6 software with the zimm plot (order 1) technique for molar-  
219 mass estimation and a differential refractive index increment of the polymer in  
220 the solvent used. A  $dn/dc$  classical value was employed for polysaccharides  
221 (0.146 mL g<sup>-1</sup>).<sup>38</sup>

## 222 **Statistical treatment**

223 One-factor ANOVA and Tukey HSD post-hoc tests were applied to establish the  
224 significance of differences between means (α = 0.05). The data were analysed  
225 with SPSS Statistics 21.0 Software for Windows (SPSS Inc., Chicago, IL).

## 226 **RESULTS AND DISCUSSION**

### 227 **Fermentations**

228 All fermentations finished in 27 days. Mixed culture of *T. delbrueckii* and *S.*  
229 *cerevisiae* (m-Td/Sc) presented a similar fermentation rate with regard to pure  
230 culture of *S. cerevisiae* (p-Sc, considered as control). In sequential culture of *T.*

231 *delbrueckii*/*S. cerevisiae* (s-Td/Sc), the addition of *S. cerevisiae* (day 6 of  
232 fermentation; Sc in graphics) produced a significant increase in the fermentation  
233 rate (Figure 1). These co-cultures with *T. delbrueckii* and *S. cerevisiae* as well as  
234 the fermentation control p-Sc were characterized by an amount of residual sugar  
235 lower to 3 g L<sup>-1</sup>, which were therefore considered as dry wines. By contrast, *T.*  
236 *delbrueckii* in pure culture presented a lower fermentative capacity, did not  
237 consume the total quantity of sugars finishing with 33.70 g L<sup>-1</sup> and 10.88 % of  
238 ethanol. It should also be highlighted the noticeably higher glycerol content  
239 found in pure and sequential of *T. delbrueckii* (7.14 and 5.28 g L<sup>-1</sup>, respectively)  
240 in comparison with mixed culture and the control (2.87 and 3.12 g L<sup>-1</sup>,  
241 respectively).

#### 242 **Purification of polysaccharides and oligosaccharides fractions**

243 Malvar wines from pure, mixed and sequential cultures with *T. delbrueckii* and *S.*  
244 *cerevisiae* native yeasts were injected on Superdex 30-HR column in order to  
245 separate polysaccharides and oligosaccharides. The first two peaks eluted  
246 between 40 and 55 min corresponds to the polysaccharide fraction, it is  
247 composed of polysaccharides rich in arabinose and galactose (PRAGs),  
248 mannoproteins (MPs) and rhamnogalacturonans type II (RG-II).<sup>6,39</sup> The  
249 polysaccharides rich in arabinose and galactose and rhamnogalacturonans come  
250 from the pecto-cellulosic cell walls of grape berries.<sup>4,7,13,16,35</sup> The mannoproteins  
251 are released from the yeast during alcoholic fermentation.<sup>35</sup> The fraction eluted in  
252 the range 56–93 min contained a complex mixture of small sugars which  
253 compose the oligosaccharides fraction of the different wines.<sup>4,13,15,16,19</sup> Because

254 the winemaking process was the same, the profiles would indicate an important  
255 influence of yeast strain employed in the fermentation process and, the type of  
256 elaboration of the wines through pure, mixed or sequential culture could also be  
257 influential. Sequential culture of *T. delbrueckii* and *S. cerevisiae* presented the  
258 highest peaks during the purification process, which could suggest that the  
259 metabolism of both yeast strains have a cumulative effect (data not shown).

### 260 **Glycosyl-linkage composition of polysaccharides fraction**

261 The wine polysaccharides fraction was methylated, hydrolysed and analyzed for  
262 partially methylated alditol acetates by GC-MS. Their corresponding glycosyl-  
263 linkage composition is given in Table 1. Neutral sugars commonly present in  
264 wine polysaccharides<sup>35</sup> can be found in Malvar wines (Table 1). The  
265 predominance of arabinose, galactose and mannose in the total colloids indicates  
266 that PRAGs from grape cell walls and mannoproteins from yeast cell walls were  
267 the major macromolecules in Malvar wines regardless of the type of inoculation  
268 used with *T. delbrueckii* and *S. cerevisiae*.

269 The major proportion of arabinosyl residues were present as terminal non-  
270 reducing position (2,3,5-tri-*O*-methyl ether) under furanose form, which may  
271 arise from both AGPs and arabinans. Arabinose linked  $\rightarrow 5$  is characteristic of  
272 branched arabinans on the rhamnogalacturonan chain of the pectins (presence of  
273 2- and 2,4-Rha).<sup>35</sup> Values obtained for  $\rightarrow 5$  arabinose linked are similar to  
274 commercial Champagne wines studies<sup>40</sup> but noticeably lower than other red  
275 wines analyzed.<sup>13,16</sup> It has been also detected arabinose linked at positions 2 and  
276 3 with similar values regarding red wines, which origin are AGPs from grapes.

277 It also contained all methyl ether corresponding to the galactose linked at  
278 positions  $\rightarrow 3$ ,  $\rightarrow 6$ ,  $\rightarrow 3,6$  and  $\rightarrow 3,4,6$  linkages that are found in AGPs.<sup>8,10,35</sup> The  
279 presence of 3,6-linked galactose indicates that type II arabinogalactans are side  
280 chains component of the RG-I in Malvar wines.<sup>8,10</sup> The ratio of 3-linked  
281 galactose to 3,6-linked galactose residues (p-Sc, 1:3.1; p-Td, 1:2.9; m-Td/Sc,  
282 1:2.7; s-Td/Sc, 1:3.0) indicated a highly-branched molecule similar to the AGP  
283 isolated from Carignan wine<sup>8</sup> or Champagne wine.<sup>40</sup> Vidal et al.<sup>3</sup> reported that  
284 the addition of a mixture of AGPs and MPs have an influence on wine  
285 astringency and fullness. However, the physicochemical and sensorial properties  
286 related with PRAGs in wines need to be investigated.

287 Mannose residues were significantly predominant in those cultures where *T.*  
288 *delbrueckii* CLI 918 strain has been used in the fermentation process. On the  
289 contrary, pure culture of *S. cerevisiae* presented lower value of mannose residues  
290 (Table 1). Significant differences in molar percentage of mannose linked in  $\rightarrow 2$ ,  
291  $\rightarrow 3$ ,  $\rightarrow 4$  and  $\rightarrow 3,4$  positions as well as in non-reducing terminal were observed,  
292 particularly for pure culture of *T. delbrueckii* and sequential culture of *T.*  
293 *delbrueckii/S. cerevisiae* compared to pure culture of *S. cerevisiae*. The glycosyl-  
294 linkage compositions of mannoproteins isolated from the Malvar wine inoculated  
295 by pure culture of *S. cerevisiae* is in good agreement with accepted structure for  
296 glycosidic moiety of mannoproteins previously isolated from a Carignan red  
297 wine.<sup>35</sup> The structure of mannoproteins in these fractions consisted of a long 6-  
298 linked backbone, highly substituted on position two with 2- and 3- linked  
299 mannose chains and attached to protein at asparagine units in the protein. These

300 structural characteristics are in agreement with the model proposed by Ballou<sup>41</sup>  
301 for mannoproteins from *Saccharomyces*, and coincided with those typically  
302 found in yeast mannoproteins<sup>42</sup> or with those mannoproteins released into the  
303 wine.<sup>35</sup>

304 Nevertheless, it is important to highlight the structural differences of  
305 mannoproteins released by *T. delbrueckii* strain in comparison with those  
306 released by *S. cerevisiae* strain. In pure and sequential cultures with *T.*  
307 *delbrueckii*, the majority of mannoproteins were composed of short 2-linked  
308 mannose chains, attached to serine and threonine residues in the protein part  
309 (29.7% and 29.3% respectively) forming highly branched molecules. The ratio  
310 between the values (Table 1) exhibited for 2-linked mannose and terminal  
311 mannose in pure culture of *T. delbrueckii* presented a value of 4, therefore the  
312 chains of mannoproteins released by this non-*Saccharomyces* strain contain 4  
313 residues of mannose linked in  $\rightarrow 2$  for a terminal mannose. Mannose linked at 4-  
314 position has only been observed in pure culture of *T. delbrueckii* (Table 1), this  
315 2,3,6-Man corresponds to the first mannose unit that it is 4-linked to the di-*N*-  
316 acetylchitobiose unit.<sup>41</sup> In pure culture of *S. cerevisiae* (p-Sc) and in pure culture  
317 of *T. delbrueckii* (p-Td) the structure of mannoproteins released into the wine are  
318 different (Table 1).

319 Besides, it was also observed in co-cultures that the secreted mannoproteins will  
320 not be the same according to whether they are inoculated at the same time (m-  
321 Td/Sc: mixed culture of *T. delbrueckii* and *S. cerevisiae*) or in a sequential  
322 manner (s-Td/Sc: sequential culture of *T. delbrueckii* and *S. cerevisiae*). In mixed

323 culture (m-Td/Sc), mannoproteins have a glycosidic structure similar to that  
324 obtained with a pure culture of *S. cerevisiae* (p-Sc). And in sequential culture (s-  
325 Td/Sc), the structure of mannoproteins is similar to that of the pure culture of *T.*  
326 *delbrueckii* (p-Td) (Mannose linked in 2-position, Table 1). Furthermore, both  
327 pure cultures presented a different ability to release polysaccharides into the  
328 wine, showing higher ratio of polysaccharide release for *T. delbrueckii* than *S.*  
329 *cerevisiae*, these results are in agreement with the results obtained by Domizio et  
330 al.<sup>25</sup> In the case of sequential culture, the polysaccharide release from *T.*  
331 *delbrueckii* CLI 918 could be explained as the result of actively growing cells of  
332 this non-*Saccharomyces* yeast strain before the inoculation of *S. cerevisiae* CLI  
333 889. In the case of mixed culture of *T. delbrueckii* and *S. cerevisiae*, the  
334 fermentation process seems to be dominated by *S. cerevisiae* as the results were  
335 similar to the control (p-Sc). In reference to polysaccharides production in mixed  
336 cultures, Cominiti et al.<sup>43</sup> observed a significant increase in the polysaccharides  
337 content only in the mixed fermentations of *T. delbrueckii/S. cerevisiae* at the  
338 inoculation ratios of 100:1 and 10,000:1. Other authors,<sup>44</sup> using a spectroscopic  
339 approach to evaluate the polysaccharide/mannoprotein ratio has found  
340 differences depending on the yeast strain or species used.

341 Moreover, it is well known the relation of MPs with the retention of aromatic  
342 compounds and the increase of body and mouth feel in wines.<sup>3,45</sup> After physico-  
343 chemical, aromatic and sensorial analysis of these wines, pure culture of *T.*  
344 *delbrueckii* and sequential culture of *T. delbrueckii* and *S. cerevisiae* contributed  
345 to increase the complexity and quality in the final wines. Specifically, sequential

346 culture was distinguished for their higher concentration of  $\beta$ -phenylethyl alcohol  
347 (rose aroma) and larger contents of esters such as 2-phenylethyl acetate, ethyl  
348 isovalerate and ethyl hexanoate. Sensorial analysis was carried out by tasting  
349 panel, tasters valued pure and sequential cultures of *T. delbrueckii* as the best  
350 ones due to their fruity and flowery aroma, higher aroma intensity and overall  
351 quality.<sup>32</sup>

352 **SEC-MALLS analysis of polysaccharides fractions from Malvar wines**  
353 **elaborated with *T. delbrueckii* and *S. cerevisiae* strains**

354 The polysaccharides relative index elution profiles show three principal  
355 populations (Figure 2). The concentration signal peaks are in the ranges 14.0–  
356 16.7 minutes for first population (P1), 16.7–18.8 minutes for second population  
357 (P2) and 18.8–20.5 minutes for third population (P3) (Figure 2, DRI signal). The  
358 molar mass of the eluting molecules decreased with increased elution volume in  
359 agreement with the normal size exclusion separation mechanism (Figure 2,  $M_w$   
360 signal). Different molecular parameters as molar mass, polydispersity index  
361 ( $M_w/M_n$ ) and intrinsic viscosity ( $[\eta]$ ) were measured in studied wines (Table 2).  
362 The molar mass appeared considerably higher for pure culture of *T. delbrueckii*  
363 in all three populations (P1: 682 000 g/mol; P2: 133 500 g/mol; P3: 33 300  
364 g/mol) in comparison with other cultures. This result was in good agreement to  
365 González-Royo et al.,<sup>26</sup> that described the presence of three populations of  
366 polysaccharides ( $HM_w$ ; 144–1,000 kDa;  $IM_w$ ; 40–144 kDa;  $LM_w$ ; 5–40 kDa) in  
367 white wine fermented by sequential inoculation with *T. delbrueckii* and *S.*  
368 *cerevisiae*. The polydispersity index ( $M_w/M_n$ ) was in general lower in third

369 populations (P3). All first populations (P1) showed higher values than values  
370 previously reported in sparkling red wines.<sup>7</sup> These first populations (P1) (14.0–  
371 16.7 minutes) mainly corresponds to mannoproteins which molecular weights  
372 have been defined in the range from 50 000 to 560 000 g/mol.<sup>35</sup> The intrinsic  
373 viscosity was notably lower for pure and sequential cultures of *T. delbrueckii* in  
374 the first population (P1), it may be related to the higher proportion of branched  
375 mannoproteins found in these two cultures. Previous publications established the  
376 influence of polysaccharides degree of branching on the intrinsic viscosity.<sup>46</sup>  
377 The molar mass distribution analysis of the polysaccharides fractions from  
378 Malvar white wines elaborated with *T. delbrueckii* and *S. cerevisiae* yeasts is  
379 shown in Figure 3. Regarding these data, six delimited ranges among different  
380 wines can be observed (Molar mass range: range 1 = 2 500–20 000 g/mol; range  
381 2 = 20 000–100 000 g/mol; range 3 = 100 000–250 000 g/mol; range 4 = 250  
382 000–500 000 g/mol; range 5 = 500 000–1 000 000 g/mol; range 6 = 1 000 000–  
383 10 000 000 g/mol). Previous six ranges have been selected due to their  
384 correspondence with values obtained from different polysaccharides families by  
385 SEC analysis: RG-II monomer,  $M_w = 5\ 000$  g/mol; RG-II dimer,  $M_w = 10\ 000$   
386 g/mol;  $MP_{0c}$ ,  $M_w = 58\ 000$  g/mol;  $AGP_2$ ,  $M_w = 165\ 000$  g/mol;  $MP_{0a}$ ,  $M_w = 350$   
387 000 g/mol;  $MP_3$ ,  $M_w = 1\ 000\ 000$  g/mol.<sup>7,35</sup> Also,  $AGP_0$  with a molar mass  
388 around 145 000 g/mol was found in Carignan red wine.<sup>35</sup>  
389 In pure culture of *T. delbrueckii* polysaccharides fractions, 0% of mass can be  
390 observed in range 1 which corresponds to polysaccharides like mannoproteins of  
391 small molar mass (and mainly RG-II in the case of red wines). However, values

392 in the range 1 in pure culture of *S. cerevisiae* (11%), mixed (8%) and sequential  
393 (10%) combinations presented values below the described in red wine.<sup>7</sup> Obvious  
394 differences are also observable concerning the ranges 2 and 3. Pure culture of *T.*  
395 *delbrueckii* showed the highest values in the range 2 followed by mixed culture,  
396 pure culture of *S. cerevisiae* and sequential culture (Figure 3). On the contrary,  
397 this sequential culture presented higher percentage of mass in range 3, then pure  
398 culture of *T. delbrueckii*, mixed culture and pure culture of *S. cerevisiae*. In  
399 contrast, the percentage of mass in range 4, range 5 and range 6 were notably  
400 similar between four different types of cultures (Figure 3).

#### 401 **Oligosaccharide composition**

402 There are no published studies about the glycosyl composition of  
403 oligosaccharides in Malvar varietal wines. In addition, this is the first time that  
404 the glycosyl composition and characteristic ratios of oligosaccharides in pure,  
405 mixed and sequential cultures with the native yeasts *T. delbrueckii* CLI 918 and  
406 *S. cerevisiae* CLI 889 has been analysed, showing the corresponding results in  
407 Table 3. The wines studied contain most of the sugars which take part in the  
408 composition of wine carbohydrates.<sup>4,7,16,35</sup> These sugars are mainly rhamnose,  
409 arabinose, galactose, xylose, galacturonic acid and glucuronic acid coming from  
410 the pecto-cellulosic cell walls of grapes. The presence of xylose, glucuronic and  
411 4-*O*-Me glucuronic acid residues indicated that traces of hemicellulose might be  
412 solubilized from grape berry cell walls.<sup>13,47</sup> Other sugars such as mannose and  
413 glucose are released from yeast polysaccharides.

414 In our work, differences were observed in the predominance of several  
415 oligosaccharides between the types of cultures. However, the predominant  
416 oligosaccharides were glucose (20.0–40.9%), galacturonic acid (14.5–26.8%),  
417 xylose (11.1–15.3%), mannose (10.3–15.3%), arabinose (8.2–10.1%) and  
418 galactose (4.3–7.9%). 4-*O*-Me glucuronic acid (2.8–3.8%), rhamnose (1.8–2.9%)  
419 and xylitol (1.9–2.5%) were also found in all cultures but in lower quantities. The  
420 smallest quantities of oligosaccharides corresponded to glucuronic acid (1.1–  
421 1.8%) and fucose (1.4–1.6%) in agreement with results obtained in red and white  
422 still wines<sup>4,13,14</sup> and sparkling wines.<sup>7</sup> As shown in Table 3, glucose content  
423 released into the medium was higher (40.9%) in pure culture of *S. cerevisiae*  
424 (culture considered as control) compared to the other samples. It could be  
425 possible that strains used in this work release a small part of their underlying  
426 layer composed of  $\beta$ -glucans, in addition to the external mannoprotein layer.<sup>20</sup>  
427 It could be highlighted the galacturonic acid content in pure culture of *T.*  
428 *delbrueckii*. The high value of galacturonic acid has been reported in red wines  
429 before, and it has been explained by differences in the pectin composition and in  
430 the natural pectinase activities present in grape skins.<sup>4,7,13,16</sup> In our case, it could  
431 be possible that the pectinase activity showed by *T. delbrueckii* CLI 918 strain<sup>28</sup>  
432 also influence this galacturonic acid value. It is well known that pectinase  
433 enzymes have a notable influence on technological and sensorial properties of  
434 wines.<sup>48</sup>  
435 Mannose residues were higher in wines elaborated with different combinations of  
436 *T. delbrueckii* and *S. cerevisiae*, in mixed and sequential inoculations (Table 3).

437 Mannose and glucose residues are mainly released from yeast cell walls, our  
438 findings regarding these two oligosaccharides are similar to those found by  
439 Quijada-Morín et al.<sup>4</sup> and higher than obtained by others authors,<sup>7,13</sup> probably  
440 due to differences in maturity stages between cultivars at time of the harvest or to  
441 different winemaking conditions.

442 The oligosaccharides coming from grape cell walls presented similar proportions  
443 in respect to those described in Chardonnay and Grignolino wines,<sup>14</sup> with  
444 exception of galacturonic acid and mannose content that was considerably higher  
445 in Malvar wines. Oligosaccharides such as arabinose, rhamnose and galactose  
446 was larger in Carignan,<sup>13</sup> Cabernet Sauvignon, Syrah and Monastrell<sup>16</sup> red wines  
447 in comparison with Malvar white wines. It could be related to longer contact  
448 between grape skins and must during the elaboration of red wines than during the  
449 production of white ones.

450 In order to know the oligosaccharide sugar structures, several characteristic ratios  
451 have been calculated (Table 3): arabinose to galactose (Ara/Gal), rhamnose to  
452 galacturonic acid (Rha/Gal A), arabinose+galactose to rhamnose (Ara+Gal/Rha)  
453 and mannose to glucose (Man/Glc).

454 The ratio Ara/Gal is characteristic of the wine PRAGs.<sup>38,47</sup> The value of this ratio  
455 has been previously described in red wines, close to 1.<sup>48</sup> Other authors obtained  
456 ratios 2-fold higher in Carignan and Merlot red wines<sup>13</sup> and in Tempranillo  
457 sparkling wines.<sup>7</sup> In our work, this ratio showed significantly lower values in the  
458 case of sequential culture of *T. delbrueckii* and *S. cerevisiae* and pure culture of  
459 *T. delbrueckii* compared to the control (Table 3). The higher ratio Ara/Gal in *S.*

460 *cerevisiae* pure culture and mixed culture suggests a release of arabinose or  
461 oligosaccharides rich in arabinose coming from pectic framework. By contrast, it  
462 could be suggested that sequential inoculation produced a slight degradation of  
463 PRAG structures. This PRAG degradation has been described previously during  
464 post maceration and malolactic fermentation<sup>49</sup> and during wine aging of lees by a  
465 partial dearabinosylation.<sup>47</sup> Moreover, champagne wines presented a much lower  
466 ratio Ara/Gal (0.18) in comparison with red wines.<sup>40</sup>

467 The Rha/Gal A ratio provides information on the relative richness of the wine  
468 oligosaccharides in homogalacturonans versus rhamnogalacturonans.<sup>50</sup> There is a  
469 slight difference in the sequential culture. Although, these low ratios (between  
470 0.1–0.2) for all the samples would suggest homogalacturonan predominance in  
471 oligosaccharides from Malvar wines. The results obtained were lower than those  
472 found in red still wines<sup>4,13,16,19</sup> but higher than results obtained in red sparkling  
473 wines.<sup>7</sup> This apparent discrepancy could be explained by the fact that grape  
474 variety could impact on wine oligosaccharide structure, as demonstrated by  
475 Apolinar-Valiente et al.<sup>16</sup>

476 It is assumed that most of the Ara and Gal residues are associated with pectin  
477 hairy regions. Therefore, the (Ara+Gal) to rhamnose ratio was calculated to  
478 estimate the relative importance of the neutral side chains to the  
479 rhamnogalacturonan backbone. This ratio was considerably higher in pure  
480 culture of *S. cerevisiae* in comparison with pure culture of *T. delbrueckii*, mixed  
481 and sequential cultures (Table 3). It might be concluded that Malvar white wines  
482 elaborated from different *T. delbrueckii*/*S. cerevisiae* combinations contain more

483 structures from the hairy regions of pectins (rhamnogalacturonan-like structures  
484 carrying neutral lateral chains) as a result of breakdown of grape cell wall berries  
485 by pectinases. This degradation could be favoured by pectinase activity presented  
486 by *T. delbrueckii* CLI 918 strain.<sup>28</sup> Different values for this ratio have been found  
487 in other grape varieties, 2.8 and 3.1 in Carignan and Merlot oligosaccharides;<sup>13</sup>  
488 2.8 for Syrah oligosaccharides, 4.7 in Cabernet Sauvignon grape variety and 5.1  
489 in Monastrell variety.<sup>16</sup> Also, Martínez-Lapuente et al.<sup>7</sup> showed the change of  
490 (Ara+Gal)/Rha ratio in Tempranillo sparkling wines during the aging.

491 The Man/Glc ratio has been related with the effectiveness of mannoproteins for  
492 protein stabilization in white wines, being more effective when this proportion is  
493 higher.<sup>45</sup> In our case, glucose was the largely major residue sugar (20.0–40.9%)  
494 whereas mannose represented smaller proportions (10.3–15.3%). Glucose is the  
495 prevalent sugar in grape berries<sup>9</sup> since it is the main component of cellulose and  
496 hemicellulosic xyloglucans. Furthermore, the presence of glucose in wines may  
497 also be related to microbial cell walls (*Botryotinia fuckeliana*, *Oenococcus oeni*)  
498 or condensed anthocyanins.<sup>39</sup> In this work, grapes were harvested in good  
499 sanitary conditions, Malvar white grapes did not contain anthocyanins, and  
500 malolactic fermentation was not conducted. Therefore, the glucose content in  
501 these Malvar wines would come from yeast glucans released during the  
502 fermentation. Because the different chemical composition indicated by the  
503 Man/Glc ratio for oligosaccharides could determine a different functional effect  
504 on the wine, further analyses will be necessary to determine a possible  
505 correlation on the wine attributes derived from the different mannoprotein

506 released through pure, mixed or sequential culture of *T. delbrueckii* CLI 918 and  
507 *S. cerevisiae* CLI 889.

508 Taking into account that the grape variety, maturity stage and grape processing  
509 were equal, the results of this study highlight that yeast strain and type of  
510 inoculation have a significant impact on wine carbohydrate composition and  
511 structure. Regarding the composition of polysaccharides fraction, mannose  
512 residues from MPs were significantly predominant in those cultures where *T.*  
513 *delbrueckii* was present in the fermentation process, whereas galactose residues  
514 from PRAGs presented higher values when pure culture of *S. cerevisiae* was  
515 employed, indicating that *S. cerevisiae* released less mannoproteins than *T.*  
516 *delbrueckii*. Concerning the molecular parameters, the molar mass appeared  
517 considerably higher for pure culture of *T. delbrueckii* in comparison with other  
518 cultures, appearing also variations with regard to the intrinsic viscosity  
519 depending on the population observed by RI technique. Moreover, the molar  
520 mass distribution of the polysaccharides fractions from Malvar white wines also  
521 showed obvious changes relied on the yeast strain and type of inoculation used.  
522 As regards the polysaccharide fractions in pure culture of *T. delbrueckii*, 0% of  
523 molar mass can be observed in the range between 2 500 and 20 000 g/mol  
524 whereas the control (p-Sc) and the co-cultures presented values between 8.4 and  
525 10.9 % for this range. Clear differences were also observable in the range  
526 between 20 000 and 100 000 g/mol and the range between 100 000 and 250 000  
527 g/mol. The analysis of Malvar wines has also revealed that the oligosaccharide

528 composition and structure could be significantly influenced by the types of  
529 cultures.

530 In summary, our results provide relevant information about the polysaccharides  
531 and oligosaccharides released from cell wall of Malvar grapes and the  
532 carbohydrates released from cell wall of different yeast strains through pure,  
533 mixed or sequential cultures of *T. delbrueckii* CLI 918 and *S. cerevisiae* CLI 889.  
534 These findings, about the use of different types of cultures, aim to improve the  
535 quality of wines from D.O. "Vinos de Madrid", highlighting especially sequential  
536 cultures to produce larger amounts of mannoproteins enhancing the complexity  
537 and quality of Malvar wines. Analysis should be carried out to deepen our  
538 knowledge concerning the capacity of *Saccharomyces* and non-*Saccharomyces*  
539 yeast strains to release mannoproteins, which could be considered a selection  
540 criterion for wine elaboration because of their reported contribution to wine  
541 quality.

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551 **Notes**

552 The authors declare no competing financial interest.

553 **ABBREVIATIONS USED**

554 PRAGs, polysaccharides rich in arabinose and galactose; AGs, type II  
555 arabinogalactans; AGPs, type II arabinogalactan-proteins; RG-I,  
556 rhamnogalacturonans type I; RG-II, rhamnogalacturonans type II; MPs,  
557 mannoproteins; TMS, per-O-trimethylsilylated methyl glycoside; HRSEC, high-  
558 resolution size-exclusion chromatography; GC-EI-MS, gas chromatography  
559 electron ionization mass spectrometry; SEC-MALLS, size exclusion  
560 chromatography-multiangle laser light scattering.

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Table 1. Glycosyl-Linkage Composition (mole percentage) of polysaccharides fractions isolated from Malvar wines elaborated with different types of inoculation of the yeast strains *T. delbrueckii* CLI 918 and *S. cerevisiae* CLI 889. Values are the mean  $\pm$  SD of fermentations.

Methyl ether	Linkage	p-Sc <sup>b</sup>	p-Td <sup>b</sup>	m-Td/Sc <sup>b</sup>	s-Td/Sc <sup>b</sup>
234 Rhamnose	Terminal	2.9 $\pm$ 0.8	1.3 $\pm$ 0.1*	2.2 $\pm$ 0.4	1.9 $\pm$ 0.2*
34 Rhamnose	2-Linked	1.8 $\pm$ 0.5	0.8 $\pm$ 0.2*	1.4 $\pm$ 0.4	1.1 $\pm$ 0.6
3 Rhamnose	2,4-Linked	0.4 $\pm$ 0.1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0*
Total rhamnose <sup>a</sup>		5.1 $\pm$ 1.4	2.4 $\pm$ 0.2*	3.8 $\pm$ 0.2	3.1 $\pm$ 0.8*
235 Arabinose	Terminal furanose	13.3 $\pm$ 3.3	11.0 $\pm$ 0.7	15.9 $\pm$ 3.5	12.2 $\pm$ 2.2
25 Arabinose	3-Linked	1.6 $\pm$ 0.4	0.6 $\pm$ 0.1*	0.2 $\pm$ 0.0*	0.6 $\pm$ 0.2*
35 Arabinose	2-Linked	0.4 $\pm$ 0.1	0.4 $\pm$ 0.0	0.2 $\pm$ 0.0	0.2 $\pm$ 0.1
23 Arabinose	5-Linked	0.4 $\pm$ 0.2	0.7 $\pm$ 0.1	0.7 $\pm$ 0.1	0.9 $\pm$ 0.3
Total arabinose <sup>a</sup>		15.7 $\pm$ 3.9	12.7 $\pm$ 0.9	17.0 $\pm$ 3.6	13.9 $\pm$ 2.8
2346 Galactose	Terminal	3.3 $\pm$ 0.4	2.5 $\pm$ 0.6	3.0 $\pm$ 0.8	2.8 $\pm$ 0.6
234 Galactose	6-Linked	8.1 $\pm$ 0.6	8.8 $\pm$ 0.1	8.1 $\pm$ 0.1	7.1 $\pm$ 0.8*
246 Galactose	3-Linked	5.6 $\pm$ 0.3	5.6 $\pm$ 0.1	5.9 $\pm$ 1.2	4.3 $\pm$ 0.9
24 Galactose	3,6-Linked	17.7 $\pm$ 1.3	16.3 $\pm$ 0.8	16.4 $\pm$ 2.1	13.1 $\pm$ 0.8*
2 Galactose	3,4,6-Linked	4.2 $\pm$ 0.6	3.5 $\pm$ 0.4	3.2 $\pm$ 0.4	2.7 $\pm$ 0.7*
Total galactose <sup>a</sup>		38.9 $\pm$ 2.9	36.7 $\pm$ 1.8	36.6 $\pm$ 4.0	30.0 $\pm$ 2.4*
2346 Glucose	Terminal	1.4 $\pm$ 0.5	1.0 $\pm$ 0.1	0.4 $\pm$ 0.0	0.3 $\pm$ 0.0
234 Glucose	6-Linked	1.2 $\pm$ 0.9	0.4 $\pm$ 0.1	0.4 $\pm$ 0.1	1.5 $\pm$ 0.8
Total glucose <sup>a</sup>		2.6 $\pm$ 0.4	1.4 $\pm$ 0.4	0.8 $\pm$ 0.6*	1.8 $\pm$ 0.9
2346 Mannose	Terminal	11.8 $\pm$ 0.1	7.0 $\pm$ 0.1*	12.3 $\pm$ 0.8	11.4 $\pm$ 0.4
346 Mannose	2-Linked	7.2 $\pm$ 0.8	29.7 $\pm$ 2.4*	12.3 $\pm$ 3.9*	29.3 $\pm$ 2.3*
246 Mannose	3-Linked	8.3 $\pm$ 0.4	0.0 $\pm$ 0.0*	7.8 $\pm$ 0.2	3.2 $\pm$ 0.7*
236 Mannose	4-Linked	0.0 $\pm$ 0.0	2.6 $\pm$ 0.7*	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
34 Mannose	2,6-Linked	8.8 $\pm$ 1.6	6.1 $\pm$ 0.5	8.1 $\pm$ 2.8	6.5 $\pm$ 1.7
26 Mannose	3,4-Linked	2.0 $\pm$ 0.5	2.0 $\pm$ 0.1	1.7 $\pm$ 0.2	1.3 $\pm$ 0.1*
Total mannose <sup>a</sup>		38.1 $\pm$ 3.3	47.4 $\pm$ 2.1*	42.2 $\pm$ 7.6	51.7 $\pm$ 0.3*
Ratio terminal/branched		0.9 $\pm$ 0.2	0.7 $\pm$ 0.0	1.0 $\pm$ 0.2	1.1 $\pm$ 0.2

<sup>a</sup> Relative molar percent of each parent sugar family (sum of ethers from one sugar type) within total sugars.

<sup>b</sup> p-Sc: pure culture of *S. cerevisiae*; p-Td: pure culture of *T. delbrueckii*; m-Td/Sc: mixed culture of *T. delbrueckii* and *S. cerevisiae*; s-Td/Sc: sequential culture of *T. delbrueckii* and *S. cerevisiae*.

\* Means statistically different from the control (p-Sc),  $p < 0.05$

Table 2. Parameters<sup>a</sup> obtained for the polysaccharides isolated from Malvar white wines elaborated with *T. delbrueckii* and *S. cerevisiae* strains using different type of inoculation.

Wine sample <sup>b</sup>	Peak <sup>c</sup>	$M_w$	$M_n$	$M_w/M_n$	Intrinsic Viscosity
		(g/mol)	(g/mol)		(mL/g)
p-Sc	1	539 000	422 200	1.31	43.59
	2	118 850	97 390	1.21	17.34
	3	18 975	16 595	1.14	9.36
p-Td	1	682 000	522 100	1.31	33.32
	2	133 500	108 200	1.23	16.30
	3	33 300	30 300	1.10	7.03
m-Td/Sc	1	679 350	453 600	1.50	51.99
	2	124 200	102 600	1.22	16.81
	3	26 490	22 990	1.15	8.32
s-Td/Sc	1	491 000	387 600	1.27	39.07
	2	129 100	115 550	1.12	17.99
	3	25 620	22 575	1.13	10.76

<sup>a</sup> Molar-mass distribution,  $M_w$  (Molar weight),  $M_n$  (Number-average mass),  $M_w/M_n$  (Polydispersity index), and Intrinsic viscosity ( $[\eta]$ ).

<sup>b</sup> Abbreviations related with the type of culture employed and the yeast strains are explained in Table 1.

<sup>c</sup> Peak 1: ranges 14.0 – 16.7 min (first population, P1); peak 2: ranges 16.7 – 18.8 min (second population, P2); peak 3: ranges 18.8 – 20.5 min (third population, P3).

Table 3. Glycosyl composition (mole percentage) and characteristics ratios of oligosaccharides isolated from Malvar wines elaborated with *T. delbrueckii* CLI 918 strain and *S. cerevisiae* CLI 889 strain using different inoculation strategies. Values are the mean  $\pm$  SD of fermentations.

	p-Sc <sup>b</sup>	p-Td <sup>b</sup>	m-Td/Sc <sup>b</sup>	s-Td/Sc <sup>b</sup>
Rha <sup>a</sup>	1.8 $\pm$ 0.9	2.9 $\pm$ 0.3*	2.7 $\pm$ 0.1*	2.7 $\pm$ 0.1*
Fuc	1.0 $\pm$ 0.3	1.4 $\pm$ 0.1*	1.4 $\pm$ 0.1*	1.6 $\pm$ 0.1*
Ara	8.2 $\pm$ 1.0	10.0 $\pm$ 2.2	10.1 $\pm$ 0.2	9.6 $\pm$ 0.4
Gal	4.3 $\pm$ 1.4	7.9 $\pm$ 0.4*	6.6 $\pm$ 0.4*	7.9 $\pm$ 0.3*
Glc	40.9 $\pm$ 1.1	20.0 $\pm$ 1.4*	21.6 $\pm$ 2.9*	26.4 $\pm$ 0.9*
Man	10.3 $\pm$ 0.3	12.4 $\pm$ 2.2	15.1 $\pm$ 1.6*	15.3 $\pm$ 0.8*
Xyl	11.1 $\pm$ 0.8	12.2 $\pm$ 2.8	15.3 $\pm$ 0.1*	15.1 $\pm$ 0.3*
Gal A	16.7 $\pm$ 1.8	26.8 $\pm$ 3.0*	19.9 $\pm$ 0.7	14.5 $\pm$ 1.1
Glc A	1.1 $\pm$ 0.1	1.3 $\pm$ 0.2	1.4 $\pm$ 0.1	1.8 $\pm$ 0.8
4-O-MeGlc A	2.9 $\pm$ 0.2	3.4 $\pm$ 0.1*	3.8 $\pm$ 0.0*	2.8 $\pm$ 0.0
Xylitol	1.9 $\pm$ 0.4	1.9 $\pm$ 0.3	2.1 $\pm$ 0.4	2.5 $\pm$ 0.2*
Ratio				
Ara/Gal	2.0 $\pm$ 0.4	1.3 $\pm$ 0.5*	1.6 $\pm$ 0.1	1.2 $\pm$ 0.1*
Rha/Gal A	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.2 $\pm$ 0.0*
(Ara+Gal)/Rha	7.9 $\pm$ 2.8	6.2 $\pm$ 0.4	6.2 $\pm$ 0.4	6.5 $\pm$ 0.4
Man/Glc	0.2 $\pm$ 0.0	0.6 $\pm$ 0.1*	0.7 $\pm$ 0.2*	0.6 $\pm$ 0.1*

<sup>a</sup> Rha, Rhamnose; Fuc, Fucose; Ara, Arabinose; Gal, Galactose; Glc, Glucose; Man, Mannose; Xyl, Xylose; Gal A, Galacturonic acid; Glc A, Glucuronic acid; 4-O-MeGlc A, 4-O-methyl Glucuronic acid.

<sup>b</sup> Abbreviations related with the type of culture employed and the yeast strains are explained in Table 1.

\* Means statistically different from the control (p-Sc),  $p < 0.05$

## FIGURE CAPTIONS

Figure 1. Fermentation kinetics of pure (p), mixed (m) and sequential (s) cultures in Malvar must with *T. delbrueckii* (Td) and *S. cerevisiae* (Sc) yeast strains. Values are the means from triplicate fermentations.

Figure 2. SEC-MALLS chromatograms and weight-average molar mass distributions of the polysaccharide fraction in pure (p), mixed (m) and sequential (s) cultures made with *T. delbrueckii* (Td) and *S. cerevisiae* (Sc) strains. Molar weight distribution ( $M_w$ ; g/mol; continuous line) and refractive index (DRI; relative scale; dashed line).

<sup>a</sup> Peak 1: ranges 14.0 – 16.7 min (first population, P1); peak 2: ranges 16.7 – 18.8 min (second population, P2); peak 3: ranges 18.8 – 20.5 min (third population, P3).

Figure 3. Distribution analysis determined by light scattering ( $dn/dc = 0.146$  mL/g) of polysaccharides fractions isolated from Malvar white wines elaborated with *T. delbrueckii* and *S. cerevisiae* yeast strains with different type of inoculation.

<sup>a</sup> Abbreviations related with the type of culture employed and the yeast strains are explained in Table 1.

Figure 1

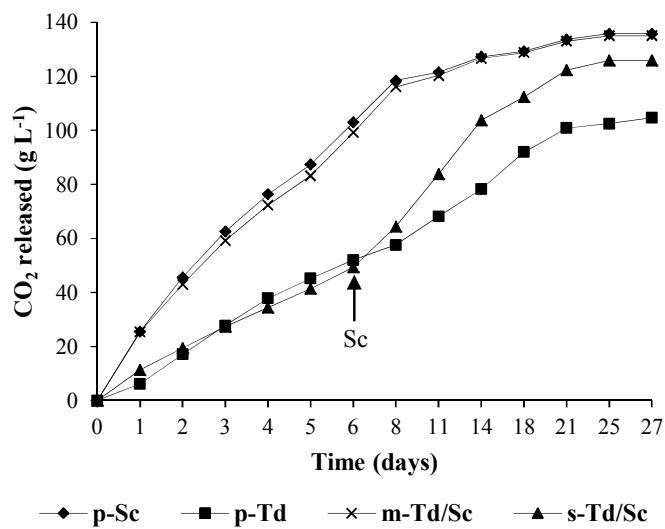


Figure 2

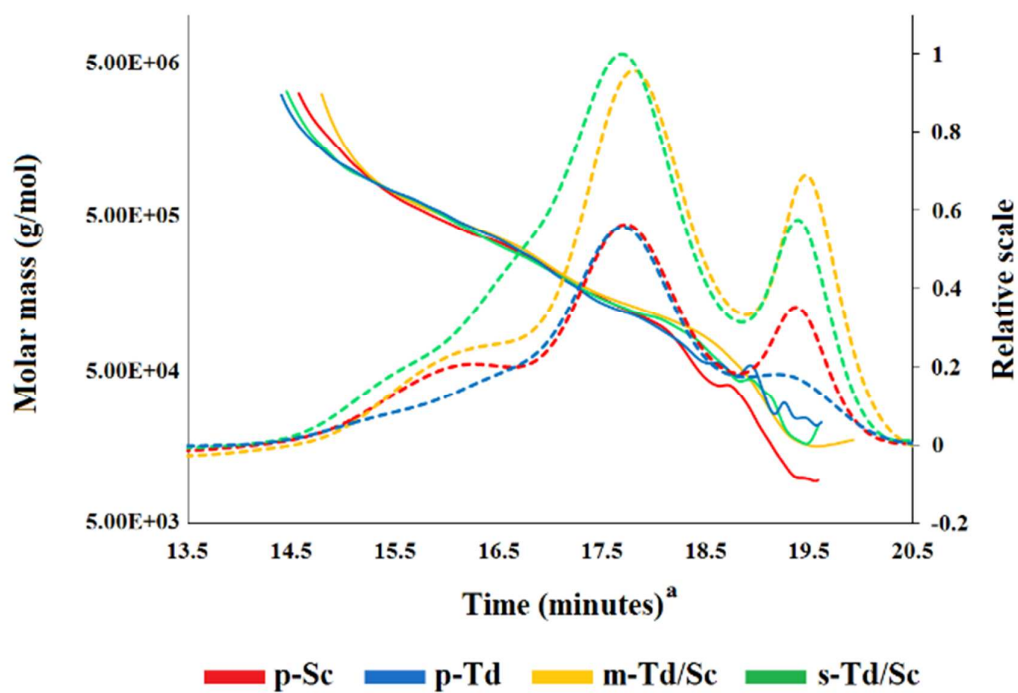
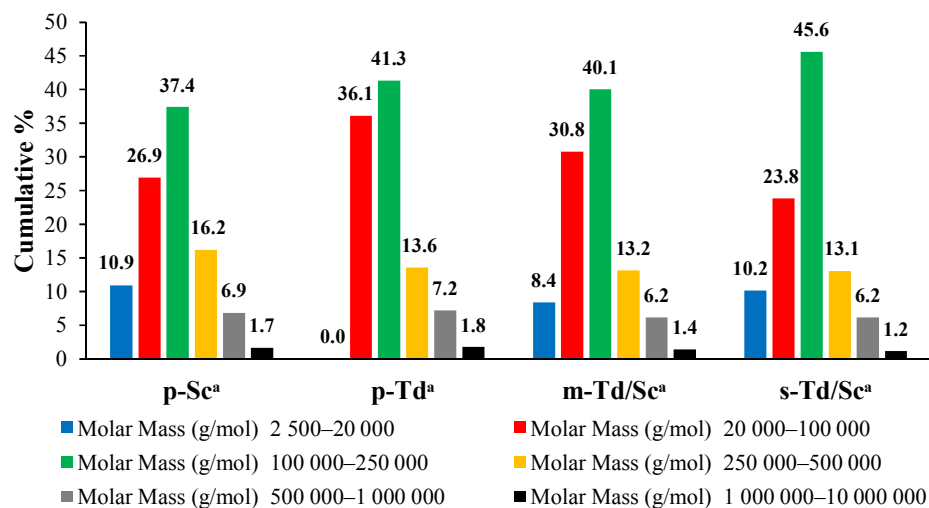


Figure 3



TOC graphic

