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1           **Structural characteristics of *Saccharomyces cerevisiae* mannoproteins:**  
2                           **impact of their polysaccharide part**

3  
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19

20           **Abstract**

21           While they have many properties of interest in enology, the structure-function relationships  
22 of mannoproteins and the part played by their polysaccharide moiety are not yet well  
23 understood. Mannoproteins (MP) extracted with  $\beta$ -glucanase from a laboratory yeast strain  
24 (WT), two of its mutants (Mnn2 with unbranched N-glycosylated chains and Mnn4 without  
25 mannosyl-phosphorylation), and an enological strain (Com) were purified and thoroughly  
26 characterized. The protein moiety of the four MPs had the same amino acid composition.  
27 Glycosyl-linkage and net charge analyses confirmed the expected differences in mutant strain  
28 MPs. MP-Com had the highest mannose/glucose ratio followed by MP-WT/MP-Mnn4, and  
29 MP-Mnn2 (13.5>5.6 $\approx$ 5.2>2.2). The molar mass dependencies of  $R_g$ ,  $R_h$ , and  $[\eta]$ , determined  
30 through HPSEC-MALLS-QELS-Viscosimetry, revealed specific conformational properties of  
31 mannoproteins related to their nature of highly branched copolymers with two branching levels.  
32 It also clearly showed structural differences between MP-Com, MP-WT/Mnn4, and MP Mnn2,  
33 and differences between two populations within the four mannoproteins.

34           **Keywords:** Mannoproteins; HPSEC-MALLS-QELS-Viscosimetry; Structural Analysis; Wine;  
35 Hyperbranched biopolymers; Structure-function relationships.

36

## 37 **1 Introduction**

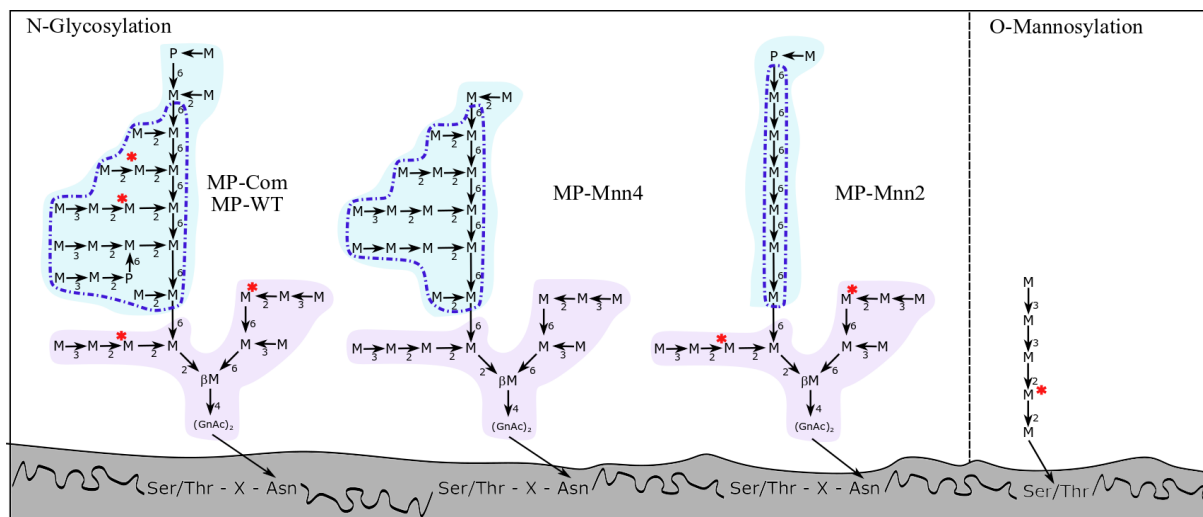
38

39 Mannoproteins represent an important family of wine polysaccharides. These  
40 proteoglycans, mainly located in the outer layer of yeast cell walls, are naturally released during  
41 wine fermentation and yeast autolysis in the aging on lees processes. In addition to the  
42 mannoproteins extracted during winemaking, the use of commercial preparations is authorized  
43 by the OIV (Organisation Internationale de la Vigne et du Vin) for specific purposes (Tartaric  
44 salts and/or protein stabilization). These commercial mannoproteins are extracted either  
45 through physico-chemical (heat, alkali, or sonication) or enzymatic methods. They must meet  
46 the specificities of the International Oenological Codex Resolution (Codex OIV-OENO 26–  
47 2004) and be composed of at least 60% in mass of polysaccharides, 70% of which must be  
48 mannose.

49 Mannoproteins account for 30-50% of the dry mass of *S. cerevisiae* cell wall, which  
50 itself represents 20-25% of the dry weight of the whole yeast cell (Aguilar-Uscanga & François,  
51 2003; Kapteyn, Van Den Ende, & Klis, 1999). They are composed of an average of 85-90%  
52 glycans, mainly D-mannose, and 10-15% protein (Orlean, 2012). The mannan chains are linked  
53 to the protein through O and N-glycosidic bonds (**Figure 1**). N-glycans are composed of an  
54 inner oligosaccharide core and a long outer chain of 50 or more  $\alpha$ -1,6-linked mannose residues,  
55 broadly branched by short chains of  $\alpha$ -1,2- mannose in extension and  $\alpha$ -1,3-mannose before the  
56 terminal (C. Ballou, 1976; Orlean, 2012). O-glycans are composed of linear structures of one  
57 to five  $\alpha$ -1,2- and  $\alpha$ -1,3-linked D-mannoses. Negatively charged mannosyl-phosphate groups  
58 can be attached to four different binding sites in the N-glycan, two in the core and two in the  
59 outer chain (**Figure 1**), and one position in O-linked oligosaccharides (Jigami & Odani, 1999).  
60 Mannoproteins are attached by their protein moiety to the inner layer of the cell wall, mainly  
61 composed of  $\beta$ -1,3-glucans and chitin through different pathways (Klis, Boorsma, & De Groot,

62 2006). Different cell-wall proteins, with different functions, have been identified (Reinhold et  
 63 al., 1997; Teparčić & Mrsá, 2013).

64



65  
 66 **Figure 1:** Schematic representation of the N-linked and O-linked carbohydrate side-chains attached to the protein moiety of mannoproteins  
 67 and the impact of  $\Delta Mnn4$  and  $\Delta Mnn2$  mutations on their structure. M: mannose residues; P: phosphate; regions in pink: core structure of the  
 68 N-glycosylation; regions in light blue: outer chain structure of the N-glycosylated side chains of the mannoproteins from the corresponding  
 69 yeast strains; region surrounded by a dashed violet line: repeated sequence of the outer chain of the N-glycosylation where the number of  
 70 repetitions can vary. \*: sites for the attachment of mannosyl-phosphate groups. The phosphorylation site in the O-linked structure is available  
 71 for all MPs except MP-Mnn4. X: Any amino acid except Proline. Adapted from Corbacho and Hernandez (Corbacho, Olivero, & Hernández,  
 72 2005).

73

74 Several works underline the various and positive functional properties of mannoproteins  
 75 in enology (Caridi, 2006; Comuzzo, Tat, Battistutta, & Tasso, 2005): adsorption of mycotoxins  
 76 (Ringot et al., 2005), stabilization against the crystallization of tartaric acid salts (Lankhorst et  
 77 al., 2017; Lubbers, Leger, Charpentier, & Feuillat, 1993) and the formation of colloidal hazes  
 78 or precipitates (Alcalde-Eon, García-Estévez, Puente, Rivas-Gonzalo, & Escribano-Bailón,  
 79 2014; Dufrechou, Doco, Poncet-Legrand, Sauvage, & Vernhet, 2015; Mekoue Nguela, Poncet-  
 80 Legrand, Siczkowski, & Vernhet, 2016; Poncet-legrand, Doco, Williams, & Vernhet, 2007;  
 81 Waters, Pellerin, & Brillouet, 1994), improvement of organoleptic properties (mouthfeel, aroma  
 82 perception, color) (Chalier, Angot, Delteil, Doco, & Gunata, 2007; Rinaldi, Gambuti, & Moio,  
 83 2012; Vidal et al., 2004), etc. These functional properties are mainly related to the

84 physicochemical interactions that develop between mannoproteins and wine constituents.  
85 However, these positive effects are variable and not fully understood yet. They are dependent  
86 on the wine composition, but more importantly, on the origin of the studied mannoproteins.  
87 Several studies indicate that the techno-functional properties of mannoproteins are dependent  
88 on their molar mass and the ratio between polysaccharide and protein moieties (Alcalde-Eon et  
89 al., 2014; Moine-Ledoux & Dubourdieu, 1999; Núñez, Carrascosa, González, Polo, &  
90 Martínez-Rodríguez, 2006; Poncet-legrand et al., 2007; Waters et al., 1994). However, neither  
91 the links between the structures of the polysaccharide and protein parts (composition, degree of  
92 branching, phosphorylation, etc.) and the properties of mannoproteins, nor their respective  
93 contributions to the latter, are yet clearly established.

94         The difficulty is that these structural features are expected to be heavily influenced by  
95 the provenance of the mannoprotein fractions: yeast strain used as a source and growth  
96 conditions, purification from wine, extraction from cell culture or lees through enzymes or  
97 physical treatments, etc. Thus, beyond the fact that only a few works have characterized the  
98 studied mannoproteins, the variability in their origin does not allow the identification of  
99 structure-function relationships (**Table 1**). Indeed, the release of mannoproteins in wines is  
100 related to yeast  $\beta$ -glucanase activities but other enzymatic activities may affect their initial  
101 structure. For example, yeast autolysis involves a huge variety of indigenous enzymes, the  
102 activities of which are no longer controlled by the dead yeast (Alexandre et al., 2001;  
103 Charpentier, 2010; Lurton, Segain, & Feuillat, 1989). In more controlled systems, enzymatic  
104 extractions are usually performed with commercial enzymes. Nevertheless, besides  $\beta$ -  
105 glucanases, other residual hydrolytic activities have been observed in these commercial  
106 cocktails, especially proteases and mannanases that can directly affect the mannoprotein  
107 structure (De Iseppi et al., 2019; Kath & Kulicke, 1999; Schiavone et al., 2014). As expected,  
108 mannoproteins extracted by physicochemical methods (heat, acid, and alkali extractions) also

109 differ in molar mass range and composition from those extracted through enzymes (De Iseppi  
110 et al., 2019; Kath & Kulicke, 1999; Moine-Ledoux & Dubourdieu, 1999; Núñez et al., 2006;  
111 Saunier, Mercereau, & Vezinhet, 1991).

112         With the final objective to better understand the structure-function relationships of  
113 mannoproteins, we focused first on the role played by the structure of their polysaccharide part  
114 and by mannosyl phosphorylation. Indeed, the structure of the polysaccharide moiety may  
115 affect the conformation of the macromolecules and their interactions in solution. Mannosyl-  
116 phosphate groups, which carry a negative charge at wine pH, may be involved in electrostatic  
117 and ionic interactions with the other positively charged wine components such as pigments and  
118 are also possible sites for hydrogen bonding. To this end, a laboratory wild-type *S. cerevisiae*  
119 strain and two of its mutants, Mnn4 and Mnn2 genetically modified to not express mannosyl-  
120 phosphorylation and  $\rightarrow 2,6$ -Man-(1 $\rightarrow$  branching linkages on the backbone of the N-  
121 glycosylation, respectively, were selected (**Figure 1**). An additional enological *S. cerevisiae*  
122 strain was also considered for comparison. The extraction method was designed to limit as  
123 much as possible the impact of yeast protease activities and thereby maintain to the utmost  
124 extent the native structure of mannoproteins. The first step, which is the subject of this article,  
125 was to extract and purify four mannoprotein pools with specific N-glycosylation and O-  
126 mannosylation structures from these strains and to thoroughly characterize them in terms of  
127 structure and conformation in solution. Our hypothesis is that the yeast strain and extraction  
128 procedure can strongly impact mannoprotein structures, whose conformations in solution are  
129 dependent their polysaccharide chains.

Extraction method	Reference	Extraction Conditions	Apparent $M_w^1$ (kDa)	Real $M_w^2$ (kDa)	Protein (mass %)	Polysacc. (mass %)	Mannose (molar %)	Glucose (molar %)	Other Sugars (molar %)	Phosphorus (mass %)
Purified after Alcoholic Fermentation or Autolysis	(Saunier et al., 1991)	Purified after AF	-	-	21	79	97.5	2.5	-	-
	(Waters et al., 1993)	Purified from red wine	60-23	-	4	96	78	13	9	-
	(Waters et al., 1994)	Purified from red wine	420	-	28.8	71.2	97.4	2.4	0.2 (GlcNAc)	-
	(Lubbers et al., 1993)	Purified/Fractionated after AF	-	-	10	90	87.7	12.3	ND	-
			-	-	12	86	83.3	16.7	ND	-
			-	-	5	90	87	13	ND	-
	(Dufrechou et al., 2015)	Purified from white wines	62	-	1.6	NS	88.8	2.6	8.5	-
	(Chalier et al., 2007)	Purified/ Fractionated after AF	225	-	11-13	NS	88-91	9-12	ND	-
			41	-	30-72	NS	65-68	32-35	ND	-
			10	-	-	NS	13	87	ND	-
	(Vidal et al., 2003) (Vernhet et al., 1996)	Purified/Fractionated from red wine according to their molecular charge	527	-	9.3	NS	91.1	5.1	3.9	-
			311	-	2.9	NS	95.9	3.6	0.6	0.34
			301	-	2.4	NS	89	3.6	6.8	0.43
337			-	1.4	NS	92.4	ND	6.9	-	
62			-	1.6	NS	88.8	2.6	8.5	0.13	
(Gonçalves et al., 2002)	Purified from white wines	51	-	3.5	NS	97.1	1.9	1.7	-	
		-	252-560	10.3	NS	100	ND	ND	-	
Physico-chemical treatments	(Saunier et al., 1991) (Moine-Ledoux & Dubourdieu, 1999)	120°C/90min	-	-	35	63.5	97.6	1.1	1.3 (GlcNAc)	1.5
		120°C/90min	-	-	4.2	93.8	92	8	ND	-
		85°C/24h	-	-	31.3	66.1	61.3	4.9	ND	-
		120°C/90min + 1min St	>250	-	7.9	92.1	84	16	ND	-
Enzymatic extractions	(Núñez et al., 2006) (De Iseppi et al., 2019)	Glucanex®	-	-	9.9	90.9	80	10.9	ND	-
		Glucanex® Zymolase®	75-250	-	1.6	98.4	93.5	6.5	ND	-
			150-250	-	1.2	98.7	69.4	30.6	ND	-
		(Moine-Ledoux & Dubourdieu, 1999)	Glucanex®	-	-	15	83.2	100	ND	ND

**Table 1:** Literature data on the characterization of mannoproteins extracted by different methods. 1Mw measured through SDS-PAGE or Pullulan Calibration. 2Mw measured by SEC-MALS). NS: Results not shown, ND: not detected by the analysis, -: analysis not performed, St: Sonication time, AF: alcoholic fermentation in model media.



## 133 2 Materials and methods

### 134 2.1 Extraction and purification of mannoproteins

#### 135 2.1.1 Yeast strains and growth conditions

136 Four different *Saccharomyces cerevisiae* strains were used: on one hand, a commercial  
137 enological strain LMD47 provided by Lallemand SAS; on the other hand a wild-type BY4742  
138 strain (MAT $\alpha$ ; ura3 $\Delta$ 0; leu2 $\Delta$ 0; his3 $\Delta$ 1; lys2 $\Delta$ 0), and its mutants  $\Delta$ Mnn4 (MAT $\alpha$ ; ura3 $\Delta$ 0;  
139 leu2 $\Delta$ 0; his3 $\Delta$ 1; lys2 $\Delta$ 0; YKL201c::kanMX4) and  $\Delta$ Mnn2 (MAT $\alpha$ ; ura3 $\Delta$ 0; leu2 $\Delta$ 0; his3 $\Delta$ 1;  
140 lys2 $\Delta$ 0; YBR015c::kanMX4). BY4742 and its mutant strains were obtained from  
141 EUROSCARF (European *Saccharomyces Cerevisiae* Archive for Functional Analysis). Yeast  
142 cells were first grown at 28°C into colonies in petri dishes on solid YEPD (1% yeast extract,  
143 2% peptone, and 2% D-glucose with 2% agar-agar). Pre-cultures were prepared by inoculating  
144 15 mL YEPD broth with a single colony and growing overnight (16-18 hours) at 28°C and  
145 under shaking. 5 mL of this first pre-culture were used to inoculate 500 mL of the second pre-  
146 culture. The yeast growth in the second pre-culture was monitored through the concentration of  
147 cells using a Coulter counter (Model Z2, Beckman-Coulter, Margency, France) fitted with a  
148 100  $\mu$ m aperture probe. This second pre-culture was used to inoculate 1 L of YEPD medium in  
149 a 5 L Erlenmeyer at a concentration of 10<sup>6</sup> cells.mL<sup>-1</sup>. Cells were harvested at the beginning of  
150 the stationary growth phase, recovered by centrifugation, and inactivated at 70°C for 20  
151 minutes. The inactivated biomass was dispersed in a YEPD medium added with 15% glycerol  
152 before being stored at -20 °C until use.

#### 153 2.1.2 Mannoproteins extraction

154 Yeast cells (1g) were washed several times with deionized water before being dispersed  
155 in 4 mL of 0.2 M acetate buffer (pH 4.5). The extraction of mannoproteins was performed using  
156 a commercial Endo- $\beta$ -1,3-glucanase enzymatic extract from *Trichoderma* sp. (E-LAMSE,

157 Megazyme, Ireland). This enzyme also contains a small residual Endo- $\beta$ -1,6-glucanase activity  
158 but no proteolytic activity (Schiavone et al., 2014). The enzyme solution (2mL) was added to  
159 the cell dispersion and the hydrolysis took place at 40 °C under magnetic stirring for 17-18  
160 hours. The reaction was stopped by enzyme inactivation at 70°C for 15 min. The suspension  
161 was centrifuged (15000 g, 15min) and the supernatant was recovered in pre-treated dialysis  
162 bags of 6-8 kDa size exclusion cut-off (Spectrum, Canada). Dialysis was performed in  
163 deionized water for 3 days with water changes three times a day. Dialyzed extracts were  
164 recovered and concentrated to a final volume of about 40 mL by evaporation at low temperature  
165 (40 °C) under vacuum.

### 166 2.1.3 Purification of the Mannoproteins from the Enzymatic Extracts

167 The purification of the enzymatic extracts was performed through Anion Exchange  
168 Chromatography assisted by an NGC Chromatographic System (BioRad, USA) equipped with  
169 both diode array and conductivity detectors. Concentrated enzymatic extracts were diluted with  
170 a 30 mM citrate-phosphate buffer (McIlvaine, 1921) at pH 3.5 until the target pH (3.5-3.6) was  
171 reached. This buffer was prepared by mixing 69.65 mL of citric acid 0.1M and 30.35 mL of  
172 Na<sub>2</sub>HPO<sub>4</sub> 0.2M before being 10-fold diluted. The solution was loaded in a prepacked HiPrep Q  
173 Sepharose XL 20 mL column (GE Healthcare, USA) equilibrated with the same buffer (eluent  
174 A). An unbound fraction was eluted with the equilibration buffer while a bound fraction (mainly  
175 proteins) was recovered through a linear ionic strength gradient: from 100 % eluent A to 100  
176 % eluent B (eluent A + NaCl 2 M) in 15 min. The unbound fraction, corresponding to the whole  
177 mannoprotein content of the enzymatic extracts, was then further purified by ultrafiltration and  
178 successive diafiltrations (up to a dilution factor 1000) to remove organic acids and salts.  
179 Ultrafiltration was performed on a 30 kDa cut-off membrane composed of regenerated cellulose  
180 from Millipore (Burlington, USA) using an Amicon® device (Burlington, USA) under the  
181 pressure of 1.5 bar and stirring. The retentates (mannoprotein pools) were recovered, filtrated

182 on 0.45  $\mu\text{m}$  hydrophilic membranes (mixed cellulose esters, Millipore), and concentrated at 10  
183  $\text{mg}\cdot\text{mL}^{-1}$  at 40  $^{\circ}\text{C}$  under vacuum. They were stored at -20  $^{\circ}\text{C}$  until further use. These  
184 mannoprotein pools are hereafter named according to the yeast strain from which they were  
185 purified: MP-Com, MP-WT, MP-Mnn4, and MP-Mnn2.

## 186 2.2 *Characterization of the pools of mannoproteins.*

### 187 2.2.1 Molecular weight distribution, static and dynamic molecular parameters.

188 The apparent molecular weight distribution of macromolecules in the enzymatic  
189 extracts, the non-retained fraction of the ion-exchange chromatography, and the purified pools  
190 were first determined by High-Performance Size-Exclusion Chromatography (HPSEC).  
191 HPSEC was performed using two serial Shodex OH-pak KB-803 and KB-805 columns (0.8 x  
192 30  $\text{cm}^2$ ; Showa Denkko, Japan) coupled with an OH-pak KB-800P guard column. 50 $\mu\text{L}$  of the  
193 samples were eluted with a 0.45  $\mu\text{m}$  filtered  $\text{LiNO}_3$  0.1 M solution (mixed cellulose ester  
194 membrane, Millipore) at a flow rate of  $1\text{mL}\cdot\text{min}^{-1}$ . The system was equipped with ERC-7512  
195 refractive index (Erma, Japan) and SPD-20A UV (280 nm, Shimadzu, Japan) detectors to  
196 distinguish between polysaccharides and proteins. The columns and UV detector were kept at  
197 room temperature while the refractive index detector was at 50  $^{\circ}\text{C}$ . The apparent molecular  
198 weight was calculated from a calibration curve established using 8 Shodex pullulan standards  
199 (P5: MW = 5 800 Da; P10: MW = 12 200 Da; P20: MW = 23 700 Da; P50: MW = 48 000 Da;  
200 P100: MW = 100 000 Da; P200: MW = 186 000 Da; P400: MW = 380 000 Da; P800: MW =  
201 853 000 Da; Showa Denko K.K.).

202 To determine the real molecular weight distribution and the static and dynamic  
203 parameters of the macromolecules, purified pools were also analyzed in an HPSEC system  
204 equipped with the same set of columns and the following set of detectors: a multi-angle (18  
205 angles) laser light (wavelength 666 nm) scattering (MALLS) where one of the angles was

206 replaced by a Quasi-Elastic Light Scattering detector (QELS) (DAWN–Heleos II from Wyatt,  
207 CA, USA), an on-line differential viscometer (VISCOSTAR II, Wyatt, CA, USA), an UV  
208 detector (280 nm) (SPD-20A, Shimadzu, Japan) and a differential refractometer (666 nm,  
209 Optilab T-Rex, Wyatt, Santa Barbara, CA, USA). Pools were dissolved in Milli-Q water at  
210  $4\text{g}\cdot\text{L}^{-1}$  and centrifuged at  $18\,000\text{ g}/15\text{ min}$  before being eluted with a filtered ( $0.1\ \mu\text{m}$  filter,  
211 mixed cellulose ester, Millipore) solution ( $0.1\ \text{M LiNO}_3 + 0.02\% \text{NaN}_3$ ) at a flow rate of  $1$   
212  $\text{mL}\cdot\text{min}^{-1}$  and  $40\ ^\circ\text{C}$ . Data were analyzed using the Astra 6.1.2 software package. An average  
213 refractive index increment value ( $dn/dc$ ) of  $0.150\ \text{mL}\cdot\text{g}^{-1}$  was used for the polysaccharide  
214 moiety,  $n$  being the refractive index of the polysaccharide solution and  $c$  the polysaccharide  
215 concentration in mass. The protein modifier method within the software was used to estimate  
216 the respective molar masses of the protein and polysaccharide parts throughout the elution  
217 profiles. To this end, the mass extinction coefficient of the protein part was determined from  
218 UV absorbency measurements at  $280\ \text{nm}$ , performed with the whole mannoprotein pools. An  
219 average value of  $1.4\ \text{mL}\cdot\text{mg}^{-1}\cdot\text{cm}^{-1}$  was determined and considered.

### 220 2.2.2 Neutral sugar analysis.

221 The neutral glycosyl-residues composition of the enzymatic extracts and mannoprotein  
222 pools was determined by gas chromatography after polysaccharides hydrolysis with  
223 Trifluoroacetic acid (Albersheim, Nevins, English, & Karr, 1967) and neutral sugar conversion  
224 in their alditol acetate derivatives (Blakeney, Harris, Henry, & Stone, 1983). Inositol and allose  
225 were added to the samples, after hydrolysis and before reduction and acetylation, as internal  
226 standards for quantification (Doco, Quellec, Moutounet, & Pellerin, 1999). The  
227 monosaccharides were identified through their retention time. GC-FID (Flame Ionization  
228 Detector) was performed by a SHIMADZU GC-2010-Plus gas chromatography system using a  
229 fused silica capillary column DB-225 ( $30\text{m} \times 0.25\ \mu\text{m} \times 0.25\ \text{mm ID}$ ) (Agilent J&W, Santa  
230 Clara, USA) with  $\text{H}_2$  as carrier gas. The Student-Newman-Keuls test was applied to an analysis

231 of variance (ANOVA) to statistically cluster similar values of composition into the same group  
232 ( $p < 0.05$ ).

### 233 2.2.3 Composition of the Glycosyl-linkages

234 The covalent links involved between glycosyl-residues of the mannoproteins within  
235 each pool were measured by GC of the partially methylated alditol acetates following the  
236 Hakomori procedure of methylation (Hakomori, 1964). Per-methylated mannoproteins (1-2  
237 mg) were submitted to pre-hydrolysis in 500  $\mu$ L of formic acid 90% at 100 °C for one hour  
238 before being cooled to room temperature and dried under dry airflow at 40 °C. Pre-hydrolyzed  
239 polysaccharides were then submitted to another hydrolysis in 2M TFA before adding inositol  
240 as an internal standard. After their reduction with NaBD<sub>4</sub>, monosaccharides were converted into  
241 their alditol acetate derivatives (Albersheim et al., 1967). Partially methylated alditol acetates  
242 were analyzed by GC-MS with a 30 m  $\cdot$ 0.25 mm OV-1 column (temperature programming:  
243 135 °C for 10 min, then 1.2 °C/min to 180 °C) with hydrogen (2 mL/min) as carrier gas. GC-  
244 MS was performed with a Hewlett-Packard HP-6890 GC coupled to a HP 5973 mass selective  
245 detector operating in the EI mode (70 eV, 34.6 IA, and  $m/z$  50-650 uma) and under control of  
246 a HP Productivity ChemStation.

### 247 2.2.4 Protein analysis.

248 The Lowry method (Lowry, Rosebrough, Lewis Farr, & Randall, 1951) was used to  
249 assess the protein content of the enzymatic extracts before their purification through ion-  
250 exchange chromatography and ultrafiltration.

251 The protein mass percentage in the purified pools of mannoproteins, and the molar  
252 percentage of each amino acid residue in it, were quantified as follows. 10 mg of freeze-dried  
253 mannoproteins were dissolved in 0.5 mL of 6N HCl and hydrolyzed at 110 °C for 24 hours.  
254 After cooling the solution, 150  $\mu$ L of norleucine solution at 2.5 M was added as an internal  
255 standard. Samples were then dried and washed twice with miliQ water and once with absolute

256 ethanol under dry airflow at 40°C. They were recovered in 750 µL of a commercial loading  
257 lithium citrate buffer at pH 2.2 and filtered with a 0.22 µm low protein binding filter  
258 (polyvinylidene fluoride - PVDF, Millipore). The quantification of the amino acids in samples  
259 was assessed by liquid chromatography with a Biochrom 30 analyzer (BIOCHROM 30,  
260 Cambridge, UK) using an ion-exchange column (Ultra-pac-8 lithium form; Amersham  
261 Pharmacia Biotech, Piscataway). As for the composition of the polysaccharide fraction, an  
262 ANOVA (SNK test,  $p < 0.05$ ) was applied to the amino acid residues composition.

### 263 2.2.5 Net charge density

264 The net charge density of the mannoproteins was measured by titration with a solution of  
265 a counter-charged polyelectrolyte using the Particle Charge Detector PCD-02 (Mütek,  
266 Germany) (Vernhet et al., 1996). The polyelectrolyte solutions used at a concentration of  $10^{-4}$   
267 N were the following: sodium polyethylenesulphonate as the anionic polyelectrolyte; and  
268 polydiallyldimethylammonium Chloride (Poly-Dadmac) as the cationic polyelectrolyte. The  
269 mannoproteins pools ( $10 \text{ mg} \cdot \text{mL}^{-1}$ ) were diluted 100 times with a solution of  $10^{-3}$  N NaCl. Their  
270 net charge was measured at 5 different pHs between 2 and 6, adjusted with  $10^{-1}$  N HCl.

## 271 3 Results and Discussion

### 272 3.1 Production of mannoproteins from yeast biomass

273 Approximately 10 g of yeast cells (dry mass) from each of the four strains were treated  
274 with the commercial Endo- $\beta$ -1,3-Glucanase. The yield of the enzymatic extraction and the  
275 composition of the enzymatic extracts (EEs) are given in **Table 2**. Mannose was the main  
276 component of all enzymatic extracts. However, all also had high amounts of glucose (especially  
277 that obtained from the Mnn2 strain) and protein (between 20 and 27%) when compared to most  
278 of the literature data (**Table 1**). For mannoproteins within the same range of molecular weight,  
279 the protein content usually represents between 4 to 12% of the dry mass, this being highly

280 dependent on their origin (purified from wine, enzymatically extracted, and physically or  
281 chemically extracted). Glucose is usually present but in lower amounts than those found in the  
282 EEs. The HPSEC analysis with RI and UV detection showed different populations in the  
283 extracts (**Supplementary Data, Figure 1**). The main peak in RI detection, eluted with a  
284 maximum of around 15 min, can be attributed to mannoproteins from the ratio between the RI  
285 and UV signals within this range. For this peak, calibration with pullulans indicated Mw  
286 distributions ranging from 50 to 500 kDa, with a maximum intensity at 150 kDa for the four  
287 MPs. By contrast, populations eluted after 17 min were likely mainly proteins or other cell  
288 components with high absorbency at 280 nm.

	YC-Com	YC-WT	YC-Mnn4	YC-Mnn2
Total biomass used (g)	11	12	12	10
<b>A</b>				
	EE-Com	EE-WT	EE-Mnn4	EE-Mnn2
Yield of enzymatic extraction (mg EE/g YC)	119.2	157.7	124.5	122.4
Protein (mass %)	21.2 ± 3.5	24.8 ± 1.4	25.1 ± 2.0	26.9 ± 8.0
Polysaccharide (mass %)	62.9 ± 0.6	60.9 ± 7.3	65.9 ± 2.9	60.9 ± 8.9
Mannose (molar %)	80.8 ± 2.4	71.1 ± 4.6	71.2 ± 0.9	52.6 ± 1.2
Glucose (molar %)	17.4 ± 2.0	27.2 ± 4.5	27.7 ± 0.8	46.1 ± 1.4
<b>B</b>				
	IEC-Com	IEC-WT	IEC-Mnn4	IEC-Mnn2
Yield of MP production (mg MP/g YC)	101.2	116.3	105.9	103.1
Protein (mass %)	16.0 ± 1.1	18.0 ± 2.2	20.0 ± 1.3	20.0 ± 5.1
Polysaccharide (mass %)	59.5 ± 3.3	65.1 ± 0.7	62.2 ± 0.4	60.6 ± 4.2
Mannose (molar %)	82.3 ± 0.2	71.5 ± 0.5	72.3 ± 0.2	56.5 ± 0.2
Glucose (molar %)	16.2 ± 0.1	26.7 ± 0.6	26.2 ± 0.1	42.2 ± 0.1
Ratio Mannose/Glucose	5.1	2.7	2.8	1.3
<b>C</b>				
	MP-Com	MP-WT	MP-Mnn4	MP-Mnn2
Yield of MP production (mg MP/g YC)	59.3	86.4	79.4	69.1
Protein (mass %)	4.3 ± 0.3 b	4.0 ± 0.3 b	3.6 ± 0.3 b	5.4 ± 0.4 a
Polysaccharide (mass %)	85.0 ± 2.3 a	77.4 ± 1.8 b	82.6 ± 2.3 ab	82.3 ± 2.2 ab
Mannose (molar %)	91.2 ± 0.0 a	83.7 ± 0.8 b	82.4 ± 1.4 b	67.3 ± 0.2 c
Glucose (molar %)	6.8 ± 0.2 d	14.8 ± 0.3 c	15.8 ± 0.6 b	30.6 ± 0.3 a
Ratio Mannose/Glucose	13.5	5.6	5.2	2.2

**Table 2:** Production yields (mg per g yeast cell biomass), protein contents, and neutral sugar compositions of the enzymatic extracts (A), of the enzymatic extracts after ion-exchange chromatography (B), and of the purified mannoprotein fractions (C). YC: yeast cells, EE: enzymatic extract, MP: Mannoproteins. Different letters indicate significant differences between samples for a given parameter (SNK's test for  $p$ -value < 0.05).

289  
290  
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292



293 Additional purification steps were applied to remove those constituents. The first step  
294 was ion-exchange chromatography. Both cation and anion-exchange chromatography were  
295 tested at different pHs, using either pH and ionic strength gradients for the elution. The best  
296 results were obtained using anion-exchange chromatography at a pH of 3.5, with an ionic  
297 strength gradient for the elution. These conditions allowed the recovery of an unbound fraction  
298 that included the mannoproteins (**Supplementary data, Figure 1**). The retained fraction was  
299 essentially composed of low molecular components and accounted for 15 to 25% of the  
300 enzymatic extract dry weight. This purification step induced a strong decrease of the UV  
301 adsorption within the low molecular weight range but both the protein and glucose contents  
302 remained high in the extracts, especially for the wild-type strain and its two mutants (**Table 2**).  
303 The exact nature of the retained fraction was not further investigated but its adsorption by anion-  
304 exchange chromatography at pH 3.5 was not expected for proteins, unless if they are very  
305 acidic. The removed components could be nucleic acids. An additional ultrafiltration step on  
306 30 kDa membranes was performed to separate the low molecular weight components from the  
307 main population. It reduced the extraction yields to values within the range 6-9% (**Table 2**).  
308 These percentages agree with theoretical values: the yeast cell wall represents about 20-25% of  
309 the cell dry mass, and mannoproteins 30-40% of the cell wall so that 60 to 100 milligrams of  
310 mannoproteins per gram of dried yeast are expected (Aguilar-Uscanga & François, 2003;  
311 Kapteyn et al., 1999).

312 The analytical methods used explained 90% of mannoprotein dry weight (**Table 2**). The  
313 four MPs had similar neutral sugar content except for a slight but significant difference between  
314 MP-Com (85%) and MP-WT (77%). By contrast, the mannose/glucose ratio varied  
315 considerably from one MP to another. The highest ratio was found for MP-Com (13.5),  
316 followed by MP-WT (5.6) and MP-Mnn4 (5.21), and then MP-Mnn2 (2.2). These results  
317 indicated differences in the polysaccharide moiety between the mannoproteins of the

318 commercial and the wild-type yeast strains. They were also consistent with the expected impact  
319 of deletions for the mannoproteins extracted from the two mutant strains compared to those  
320 from the wild strain. The mutation of the Mnn4 strain results in the absence of mannosyl  
321 phosphate groups attached to the  $\alpha$ -1,2-mannose branches of N-linked and O-Linked  
322 mannoproteins (**Figure 1**), and the mannose/glucose ratio was only slightly lower than that of  
323 MP-WT. By contrast, the mannoproteins from the Mnn2 mutant, expected to be devoid of  $\alpha$ -  
324 1,2-mannan branches, exhibited a much lower mannose content, associated with a higher  
325 glucose content. The absence of mannan ramifications and the lower quantity of mannose  
326 residues in MP-Mnn2 may be the reason for the higher protein mass percentage observed in  
327 this pool (5.4%) when compared to those with less or non-affected polysaccharide structures  
328 (MP-Com: 4.29; MP-WT: 3.98; MP-Mnn4: 3.57), and the high glucose proportion in MP-Mnn2  
329 might be a physiological response of the yeast cell to the depletion of the natural mannoprotein  
330 structure.

### 331 3.2 *Amino acid composition of the protein part*

332 The amino acid composition of the protein part of the four MPs is given in **Table 3**. They  
333 all had very similar amino acid compositions. Serine and threonine accounted together for about  
334 35% of the total amino acids. These two residues are essential for both N-glycosylation or O-  
335 mannosylation attachments of the carbohydrate chains to the protein part (Orlean, 2012).  
336 Proline (~6%), alanine (~8%), glutamine/glutamic acid (~11%), valine (~8%), and  
337 asparagine/aspartic acid (~9%) represented about 42% of the total. Asparagin is another  
338 important amino acid, involved with serine and threonine in N-glycosylation (**Figure 1**) (Lehle  
339 & Bause, 1984). Other amino acids represented about 20% percent of the total, of which less  
340 than 2% were sulfur residues (cysteine, cysteic acid). Percentages of acidic, basic, hydrophilic,  
341 and hydrophobic residues were calculated from this composition (**Table 3**). For all four MPs,

342 25% of the protein moiety is composed of acidic and basic residues, almost 40% of hydrophobic  
 343 residues, and about 38% of neutral hydrophilic residues. From these results, it could be  
 344 concluded that the main differences between the studied mannoproteins are related to their  
 345 polysaccharide part. There are only a few data on the amino acid composition of the  
 346 mannoproteins, and the latter concern mannoproteins purified from red wine (Waters et al.,  
 347 1994, 1993). Waters *et al.* found a higher % in serine and a lower % in aspartic acid, among  
 348 other minor differences. As these mannoproteins were extracted from wine, these differences  
 349 may be related to proteolytic activities.  
 350

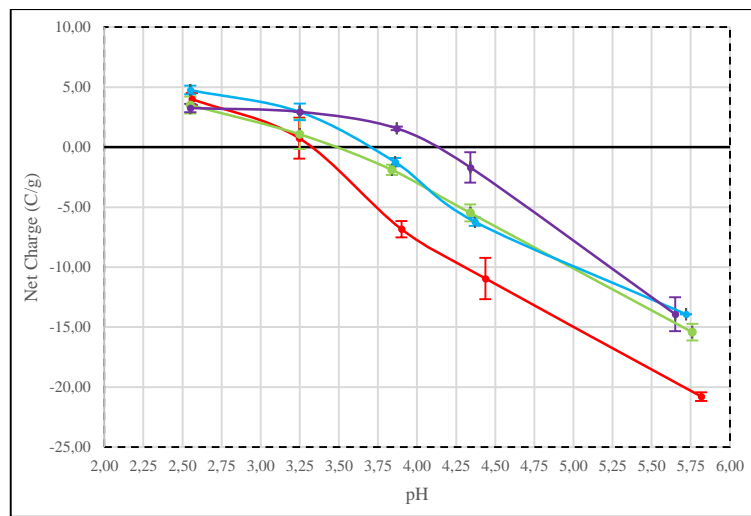
Amino acid residue	MP-Com	MP-WT	MP-Mnn4	MP-Mnn2
Cysteic acid	1.1 ± 0.8 a	1.1 ± 0.9 a	1.5 ± 0.5 a	1.2 ± 0.5 a
Alanine	8.5 ± 0.5 a	7.8 ± 0.3 a	8.0 ± 0.5 a	8.0 ± 0.4 a
Arginine	0.4 ± 0.1 b	1.0 ± 0.0 a	0.8 ± 0.3 ab	0.6 ± 0.2 ab
Aspartic acid	8.8 ± 0.2 c	9.1 ± 0.1 b	9.4 ± 0.0 a	9.2 ± 0.1 b
Glutamic acid	10.2 ± 0.6 c	10.8 ± 0.1 bc	11.1 ± 0.1 ab	11.6 ± 0.3 a
Glycine	3.3 ± 0.0 a	3.2 ± 0.4 a	3.2 ± 0.2 a	3.0 ± 0.1 a
Histidine	1.7 ± 0.1 a	1.6 ± 0.1 a	1.5 ± 0.2 a	1.3 ± 0.2 a
Isoleucine	3.4 ± 0.0 d	3.8 ± 0.1 c	4.0 ± 0.1 b	4.2 ± 0.0 a
Leucine	3.3 ± 0.2 a	3.3 ± 0.1 a	3.2 ± 0.3 a	3.2 ± 0.3 a
Lysine	3.8 ± 0.2 b	4.3 ± 0.1 a	3.9 ± 0.0 b	3.7 ± 0.1 b
Phenylalanine	1.1 ± 0.1 a	1.1 ± 0.1 a	1.2 ± 0.3 a	1.3 ± 0.3 a
Proline	6.4 ± 0.8 a	6.7 ± 0.4 a	6.0 ± 0.7 a	6.9 ± 0.4 a
Serine	12.8 ± 0.8 a	12.5 ± 0.5 a	13.0 ± 0.6 a	13.0 ± 0.5 a
Threonine	23.9 ± 0.8 a	23.5 ± 0.5 a	24.0 ± 1.2 a	23.7 ± 0.9 a
Tyrosine	1.6 ± 0.8 a	1.6 ± 1.1 a	1.0 ± 1.1 a	1.4 ± 1.0 a
Valine	9.3 ± 0.2 a	7.9 ± 0.2 b	8.1 ± 0.4 b	7.8 ± 0.3 b
Acidic residues	19.0 ± 0.4 c	19.9 ± 0.2 b	20.6 ± 0.1 a	20.7 ± 0.2 a
Basic residues	6.0 ± 0.3 b	7.0 ± 0.1 a	6.2 ± 0.6 b	5.6 ± 0.5 b
Hydrophilic residues	38.4 ± 0.9 a	37.6 ± 0.6 a	38.0 ± 0.8 a	38.1 ± 1.0 a
Hydrophobic residues	31.9 ± 1.3 a	29.9 ± 0.7 b	29.7 ± 0.6 b	30.2 ± 0.4 b

351 **Table 3:** Composition of the protein moiety of the mannoprotein pools. Molar percentage of each amino acid residue. Percentage of acidic  
 352 (glutamic and aspartic acids), basic (arginine, histidine, and lysine), hydrophilic (serine, threonine, and tyrosine), and hydrophobic (alanine,  
 353 glycine, leucine, phenylalanine, proline, and valine) residues. Different letters indicate significant differences between samples for a given  
 354 parameter (SNK's test for *p*-value < 0.05).

### 355 3.3 *Net charge density*

356 The net charge of mannoproteins is pH-dependent and results from the presence of acidic  
357 phosphate groups in their polysaccharide moiety and of basic and acidic amino acid residues in  
358 their protein part. Since the overall amino acid compositions of all four MPs are very similar,  
359 differences between the four fractions are expected to be related to different amounts of  
360 mannosyl-phosphate units in their polysaccharide moiety. The highest phosphorylation levels  
361 are expected for MP-Com and WT. The genetic modification in Y-Mnn2 affects the number of  
362 possible sites for mannosyl-phosphorylation since the N-glycosylation backbone is linear and  
363 unbranched. However, there are other possible phosphorylation sites in the core of the N-linked  
364 and in the O-linked polysaccharide structure regulated by the Mnn4 phenotype (Olivero, Mañas,  
365 & M. Hernández, 2000; Orlean, 2012). Therefore, there is no mannosyl-phosphate in MP-Mnn4  
366 so that its charge and isoelectric point (IP) will be governed by its protein moiety. The variation  
367 in the net charge density with pH is reported in **Figure 2** for the four MPs. The mannoprotein  
368 fractions had different IPs, which was consistent with different mannosyl-phosphate contents:  
369 the lowest was found for MP-Com (around 3.3), followed by MP-WT (3.5), MP-Mnn2 (3.7),  
370 and MP-Mnn4 (4.15). For pH below IP, the positive charge was weak whatever the fraction  
371 considered, in line with the low content in basic amino acids of their protein part (**Table 3**). For  
372 pH above their IP, MP-Com exhibited a higher negative charge than the others. This could  
373 reflect a higher phosphorylation degree by comparison to MP-WT. These results differ from  
374 previous ones obtained on different mannoproteins fractions purified from red wine (Vernhet  
375 et al., 1996). In this case, the purified mannoproteins all had an overall negative charge over a  
376 pH range of 2-9 and could be separated into three fractions by ion-exchange chromatography  
377 according to their charge (Vidal et al., 2003). Such separation was not possible in the present  
378 study. As already stated, this may be related to the presence of proteolytic activities during  
379 winemaking, along with  $\beta$ -glucanase ones. These proteolytic activities may be responsible for

380 different ratios between the mannosyl-phosphate groups on one hand and acidic and basic  
381 amino acid residues on the other hand.



382

383 **Figure 2:** The net charge density of the mannoprotein pools at different pHs. MP-Com (Red); MP-WT (Green); MP-Mnn4 (Violet) and MP-  
384 Mnn2 (Light Blue).

### 385 3.4 Glycosyl Linkage Analysis

386 The molar percentages of glycosyl linkages for mannose and glucose in the purified  
387 mannoprotein pools are shown in **Table 4**. MP-Com, MP-WT, and MP-Mnn4 had similar  
388 compositions, with small differences among them. They had low amounts of  $\rightarrow 6$ -Glc-(1 $\rightarrow$   
389 from  $\beta$ -1,6-glucans chains that connect mannoproteins to the yeast cell wall. These linking  
390 structures are not fully hydrolyzed during the enzymatic extraction since the enzyme used has  
391 only a small residual  $\beta$ -1,6-glucanase activity (Schiavone et al., 2014). There was  
392 approximately three times less glucose associated with MP-Com by comparison to MP-WT and  
393 MP Mnn4.  $\rightarrow 2,6$ -Man-(1 $\rightarrow$  residues are mainly related to the ramifications in the backbone  
394 of the N-glycosylation (**Figure 1**) and to the attachment sites of mannosyl-phosphate groups in  
395 the inner core and outer chains (Jigami & Odani, 1999). Their high proportions, associated with  
396 the low proportions of  $\rightarrow 6$ -Man-(1 $\rightarrow$  (non-branched mannose residues in the backbone of N-  
397 glycosylated chains) and the presence of  $\rightarrow 2$ -Man-(1 $\rightarrow$  and  $\rightarrow 3$ -Man-(1 $\rightarrow$  residues  
398 belonging to the side chains of the outer-chain part and core of N-linked oligosaccharides and

399 to the linear structure of the O-linked oligosaccharides, highlighted the highly branched  
 400 structure of the polysaccharide side chains of MP-Com, MP-WT, and MP-Mnn4 (L. Ballou,  
 401 Hernandez, Alvarado, & Ballou, 1990).  $\rightarrow$ 6)-Man-(1 $\rightarrow$  and  $\rightarrow$ 3)-Man-(1 $\rightarrow$  units were found  
 402 in higher proportions (between 33 et 42% higher) in MP-Com than in MP-WT and MP-Mnn4.  
 403 This was associated with lower proportions of  $\rightarrow$ 2)-Man-(1 $\rightarrow$  (between 13 to 15%). This  
 404 suggested differences in the branching patterns of the N-glycosylated chains between these  
 405 mannoproteins.  
 406

		MP-Com	MP-WT	MP-Mnn4	MP-Mnn2
Glycosyl					
Residue	Linkage				
2,3,4,6-Glucose	Glc-(1 $\rightarrow$	0.7	1.5	1.5	4.8
2,3,4-Glucose	$\rightarrow$ 6)-Glc-(1 $\rightarrow$	2.2	5.7	6.4	20
2,4-Glucose	$\rightarrow$ 3,6)-Glc-(1 $\rightarrow$	-	-	1.6	3.7
Total Glucose (% molar)		2.8	7.3	9.5	28.5
2,3,4,6-Mannose	Man-(1 $\rightarrow$	29.7 (30.6)	30.6 (33.0)	29.5 (32.6)	23.4 (32.7)
2,3,4-Mannose	$\rightarrow$ 6)-Man-(1 $\rightarrow$	1.2 (1.2)	0.7 (0.8)	0.8 (0.9)	20.1 (28.1)
2,4,6-Mannose	$\rightarrow$ 3)-Man-(1 $\rightarrow$	18.9 (19.4)	11.4 (12.3)	10.3 (11.4)	6.2 (8.7)
3,4,6-Mannose	$\rightarrow$ 2)-Man-(1 $\rightarrow$	21.3 (21.9)	23.5 (25.4)	22.8 (25.2)	18.3 (25.6)
2,4-Mannose	$\rightarrow$ 3,6)-Man-(1 $\rightarrow$	0.6 (0.6)	0.4 (0.4)	0.6 (0.7)	1.3 (1.8)
3,4-Mannose	$\rightarrow$ 2,6)-Man-(1 $\rightarrow$	25.2 (25.9)	25.8 (27.8)	26.5 (29.3)	1.2 (1.7)
3-Mannose	$\rightarrow$ 2,4,6)-Man-(1 $\rightarrow$	0.3 (0.3)	0.2 (0.2)	0.3 (0.3)	0.9 (1.3)
Total Mannose (% molar)		97.2 (100.0)	92.7 (100.0)	90.5 (100.0)	71.5 (100.0)
Branching Degree (DB) (%)		56	60	62	13

407 **Table 4:** Molar percentage of the glycosyl linkage residues in the polysaccharide moiety of each MP. Values in parentheses represent the  
 408 molar percentages only in mannosyl linkage residues. The branching degree of the mannan chains of the polysaccharide moiety was calculated  
 409 according to Hölter et al. (Hölter, Burgath, & Frey, 1997).

410 As expected, the strongest differences were evidenced between MP-Mnn2 and the three  
 411 other mannoproteins. MP-Mnn2 had much lower contents of branched  $\rightarrow$ 2,6)-Man-(1 $\rightarrow$ )  
 412 residues and much higher amounts in  $\rightarrow$ 6)-Man-(1 $\rightarrow$ , which reflects the lack of branching of  
 413 the outer chain of the N-glycosylated oligosaccharides. The branched residues still present in  
 414 MP-Mnn2 likely belong to the core structure, unaffected by the gene deletion. Molar  
 415 percentages of linear  $\rightarrow$ 2)-Man-(1 $\rightarrow$  and  $\rightarrow$ 3)-Man-(1 $\rightarrow$ , similar to the other three MPs, could

416 be an indication that mannoproteins in this yeast strain have higher proportions in O-linked than  
417 N-linked side chains. As already stated, MP-Mnn2 also had a higher proportion of glucose than  
418 the other mannoproteins, which is mostly related to  $\rightarrow 6$ -Glc-(1 $\rightarrow$  residues.

### 419 3.5 Molar mass distribution and molecular properties

420 The HPSEC-MALS chromatograms of the four mannoprotein pools are shown in **Figure**  
421 **4**. The elution profiles (RI and UV) are represented along with the molar mass ( $M_w$ ) profile  
422 calculated from the SLS (Static Light Scattering) measurements. All MP pools presented  
423 populations of different sizes and proportions. The HPSEC profiles showed two populations for  
424 MP-com: a small population in terms of amount with a high hydrodynamic radius ( $R_h$ ) and  
425 average molar mass, thereafter named population 1, and a predominant population of lower  
426 hydrodynamic radius and average molar mass, thereafter named population 2. A third  
427 population, eluted last, was also evidenced for MP-WT, MP-Mnn2, and MP-Mnn4. The  
428 proportions of these different populations within each mannoprotein pool, along with the static  
429 ( $M_w$ ,  $M_n$ ,  $R_g$ ) and dynamic ( $[\eta]$ ,  $R_h$ ) average parameters of populations 1 and 2, are summarized  
430 in **Table 5**. The third population detected in MP-WT, MP-Mnn2 and MP-Mnn4 only  
431 represented a low mass percentage and was mainly co-eluted with population 2. Thus, analytical  
432 results were unreliable and won't be further discussed. The relative UV signal by comparison  
433 to the RI one also indicated higher protein/polysaccharide ratios for the mannoproteins in  
434 population 1 compared to those in population 2. Assuming for the protein part a close mass  
435 extinction coefficient over the whole distribution, and considering  $dn/dc$  value of 0.185 and  
436 0.146 mL/g for proteins (Zhao, Brown, & Schuck, 2011) and polysaccharides (Ioan, Aberle, &  
437 Burchard, 2000; Denis Renard, Lavenant-Gourgeon, Ralet, & Sanchez, 2006; Rolland-Sabaté,  
438 Mendez-Montealvo, Colonna, & Planchot, 2008), respectively, it was possible to calculate for  
439 the two populations of each MP pool a protein/polysaccharide ratio and the respective molar  
440 mass of the protein and polysaccharide parts (**Figure 4 and Table 5**).

	MP-Com	MP-WT	MP-Mnn4	MP-Mnn2
<b>Total Pool</b>				
Peak Limits (min)	13 – 18.1	13 – 18.8	13 – 19.2	13 – 20
Mass Fraction (%)	100	100	100	100
Mass Recovery (%)	81	82	100	87
Protein Mass (%)	3.4	3.9	3.9	5.6
Total Mw ( $\times 10^5$ g.mol <sup>-1</sup> )	4.6 $\pm$ 0.0%	13.0 $\pm$ 0.1%	11.9 $\pm$ 0.1%	17.3 $\pm$ 0.1%
Total Mn ( $\times 10^5$ g.mol <sup>-1</sup> )	2.8 $\pm$ 0.0%	5.0 $\pm$ 0.1%	4.2 $\pm$ 0.1%	6.2 $\pm$ 0.2%
PdI (Mw/Mn)	1.6 $\pm$ 0.1%	2.6 $\pm$ 0.1%	2.8 $\pm$ 0.2%	2.8 $\pm$ 0.3%
Protein Mw ( $\times 10^5$ g.mol <sup>-1</sup> )	0.2 $\pm$ 0.9%	0.8 $\pm$ 0.5%	0.7 $\pm$ 0.5%	1.3 $\pm$ 0.4%
Protein Mn ( $\times 10^5$ g.mol <sup>-1</sup> )	0.1 $\pm$ 0.3%	0.2 $\pm$ 0.3%	0.1 $\pm$ 0.3%	0.3 $\pm$ 0.4%
Protein PdI (Mw/Mn)	2.1 $\pm$ 0.9%	4.8 $\pm$ 0.6%	5.1 $\pm$ 0.6%	4.4 $\pm$ 0.5%
R <sub>g</sub> (nm)	13.8 $\pm$ 0.3%	32.9 $\pm$ 1.3%	34.9 $\pm$ 1.3%	45.7 $\pm$ 0.5%
R <sub>h</sub> (nm)	12.1 $\pm$ 0.1%	15.8 $\pm$ 0.2%	15.3 $\pm$ 0.1%	18.0 $\pm$ 0.2%
[ $\eta$ ] (ml.g <sup>-1</sup> )	30 $\pm$ 0.3%	30 $\pm$ 0.4%	30 $\pm$ 0.3%	33 $\pm$ 0.3%
<b>Population 1</b>				
Peak Limits (min)	13 – 14.5	13 – 14.4	13 – 14.7	13 – 14.4
Mass Fraction (%)	6.6	12.1	15.1	14.6
Protein Mass (%)	5.2	6.7	6.3	8.3
Total Mw ( $\times 10^5$ g.mol <sup>-1</sup> )	21 $\pm$ 0.0%	65 $\pm$ 0.1%	53 $\pm$ 0.1%	74 $\pm$ 0.1%
Total Mn ( $\times 10^5$ g.mol <sup>-1</sup> )	19 $\pm$ 0.0%	57 $\pm$ 0.1%	41 $\pm$ 0.1%	69 $\pm$ 0.1%
PdI (Mw/Mn)	1.1 $\pm$ 0.0%	1.1 $\pm$ 0.1%	1.3 $\pm$ 0.1%	1.1 $\pm$ 0.1%
Protein Mw ( $\times 10^5$ g.mol <sup>-1</sup> )	1.1 $\pm$ 1.6%	4.5 $\pm$ 0.5%	3.5 $\pm$ 0.5%	6.5 $\pm$ 0.4%
Protein Mn ( $\times 10^5$ g.mol <sup>-1</sup> )	1 $\pm$ 1.1%	3.7 $\pm$ 0.5%	2.5 $\pm$ 0.5%	5.7 $\pm$ 0.3%
Protein PdI (Mw/Mn)	1.2 $\pm$ 2.0%	1.2 $\pm$ 0.7%	1.4 $\pm$ 0.7%	1.1 $\pm$ 0.5%
R <sub>g</sub> (nm)	24 $\pm$ 0.0%	34 $\pm$ 0.3%	34 $\pm$ 0.3%	38 $\pm$ 0.5%
R <sub>h</sub> (nm)	24 $\pm$ 0.3%	34 $\pm$ 0.2%	32 $\pm$ 0.1%	38 $\pm$ 0.1%
[ $\eta$ ] (ml.g <sup>-1</sup> )	44 $\pm$ 0.9%	39 $\pm$ 0.7%	42 $\pm$ 0.4%	46 $\pm$ 0.4%
<b>Population 2</b>				
Peak Limits (min)	15 – 18.1	15 – 17	15 – 17.5	15 – 17
Mass Fraction (%)	92.8	66.6	71.7	51.9
Protein Mass (%)	3.3	3	3	5.2
Total Mw ( $\times 10^5$ g.mol <sup>-1</sup> )	3.4 $\pm$ 0.0%	5.4 $\pm$ 0.1%	4.5 $\pm$ 0.1%	8 $\pm$ 0.2%
Total Mn ( $\times 10^5$ g.mol <sup>-1</sup> )	2.7 $\pm$ 0.0%	4.5 $\pm$ 0.1%	3.7 $\pm$ 0.1%	5.7 $\pm$ 0.2%
PdI (Mw/Mn)	1.3 $\pm$ 0.1%	1.2 $\pm$ 0.1%	1.2 $\pm$ 0.2%	1.4 $\pm$ 0.3%
Protein Mw ( $\times 10^5$ g.mol <sup>-1</sup> )	0.12 $\pm$ 1.6%	0.2 $\pm$ 0.3%	0.2 $\pm$ 0.3%	0.5 $\pm$ 0.3%
Protein Mn ( $\times 10^5$ g.mol <sup>-1</sup> )	0.08 $\pm$ 1.1%	0.1 $\pm$ 0.2%	0.1 $\pm$ 0.3%	0.3 $\pm$ 0.3%
Protein PdI (Mw/Mn)	1.4 $\pm$ 2.0%	1.4 $\pm$ 0.4%	1.3 $\pm$ 0.4%	1.7 $\pm$ 0.4%
R <sub>g</sub> (nm)	13 $\pm$ 0.5%	25 $\pm$ 0.7%	25 $\pm$ 1.3%	37 $\pm$ 0.3%
R <sub>h</sub> (nm)	11 $\pm$ 0.1%	13 $\pm$ 0.1%	12 $\pm$ 0.1%	16 $\pm$ 0.1%
[ $\eta$ ] (ml.g <sup>-1</sup> )	29 $\pm$ 0.3%	30 $\pm$ 0.2%	29 $\pm$ 0.2%	35 $\pm$ 0.2%

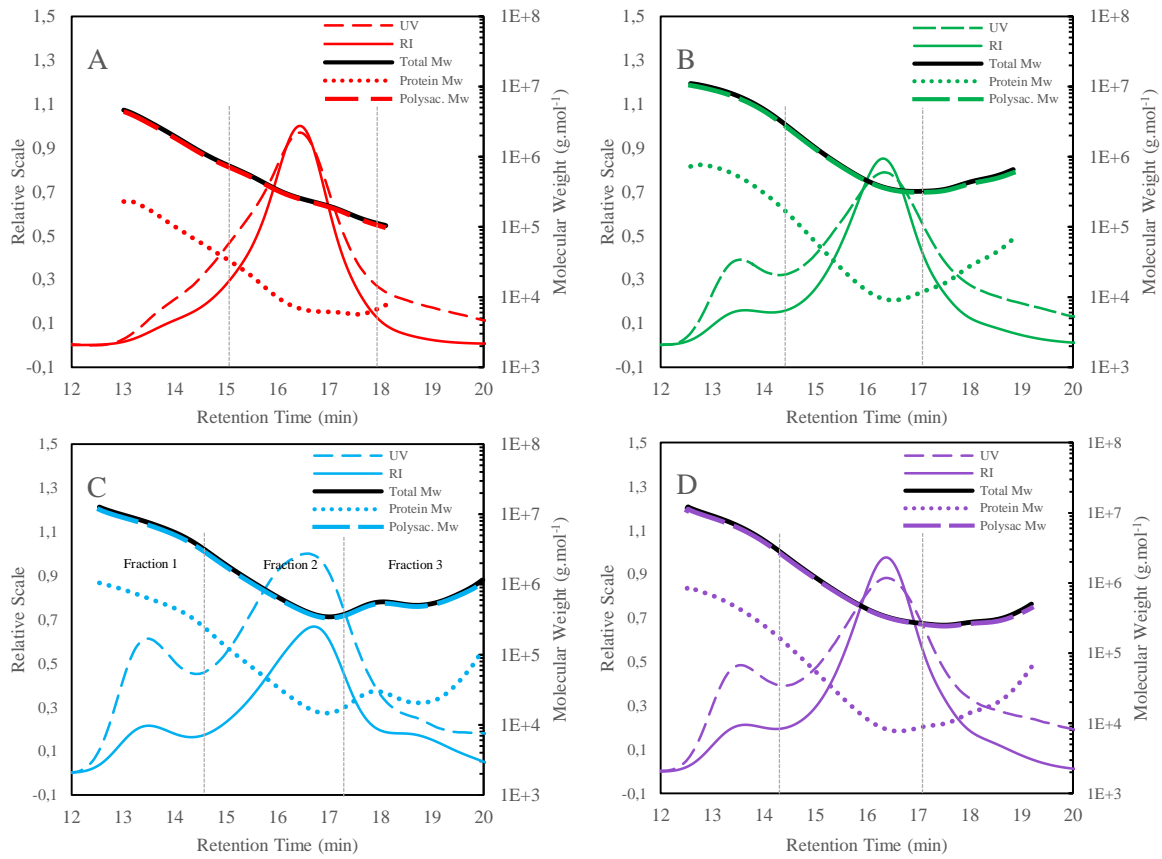
**Table 5:** Average molecular parameters of the two main populations observed in the HPSEC-MALS profiles of the four mannoprotein pools (MP-Com, MP-WT, MP-Mnn2, and MP-Mnn4). Mw: Molecular weight in mass, Mn: Molecular weight in number, PdI: Polydispersity Index, R<sub>g</sub>: Radius of Gyration, R<sub>h</sub>: Hydrodynamic radius, [ $\eta$ ]: intrinsic viscosity.

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444 The predominant populations in the four mannoprotein pools (populations 2) had average  
445  $M_w$  ranging between  $3.4 \times 10^5$  (MP-Com) and  $8 \times 10^5$  (MP-Mnn2)  $\text{g} \cdot \text{mol}^{-1}$ , and polydispersity  
446 indexes between 1.2 and 1.4. The protein moiety represented between 3 to 5% in mass of the  
447 whole macromolecules. Population 1 only represented 6.6% of MP-Com but between 12 to 15  
448 % of the other mannoprotein pools. It had much higher  $M_w$  than the population 2 (from 6 in  
449 MP-Com to 9-12 times higher in MP-WT, Mnn2, and Mnn4), associated with lower  
450 polydispersity indexes in all MP pools but MP-Mnn4. Populations 1 also had higher protein  
451 contents (between 5 to 8% in mass) than populations 2. These different proportions indicate  
452 that populations 1 are not related to aggregates of macromolecules belonging to population 2.  
453 The hydrodynamic radius of these high molecular weight populations was about twice higher  
454 than those of population 2. Although the differences in  $R_h$  between the two populations in the  
455 mannoprotein pools were relatively small, they were associated with substantial differences in  
456 molecular weight. Average  $M_w$ , determined by SLS (Static Light Scattering), ranged from  
457 460kDa (MP-Com) to 1 730 kDa (MP-Mnn2), which were higher than those indicated by  
458 calibration with pullulans (maximum values around 150kDa for all mannoprotein pools,  
459 **Supplementary Data, Figure 1**), as expected for branched structures. The real molecular  
460 weight distributions in the four MPs were also different than those observed in the literature.  
461 While MP-Com oscillated from 100 - 4 000 kDa the other three MP pools fluctuated between  
462 300 kDa and 11 000 kDa (**Figure 4**). Whereas mannoproteins purified from white wines  
463 (molecular weight also determined by MALLS) ranged from 30 to 300 kDa (Gonçalves et al.,  
464 2002). When testing different methods for mannoprotein extraction from brewery and bakery  
465 *S. cerevisiae* strains, Kulicke & Kath observed that the highest molecular weights ( $M_w$  of 410  
466 kDa determined through HPSEC-MALS) were obtained when mannoproteins were extracted  
467 with commercial  $\beta$ -glucanases completely free of protease activity (Kath & Kulicke, 1999), as  
468 in the present study.

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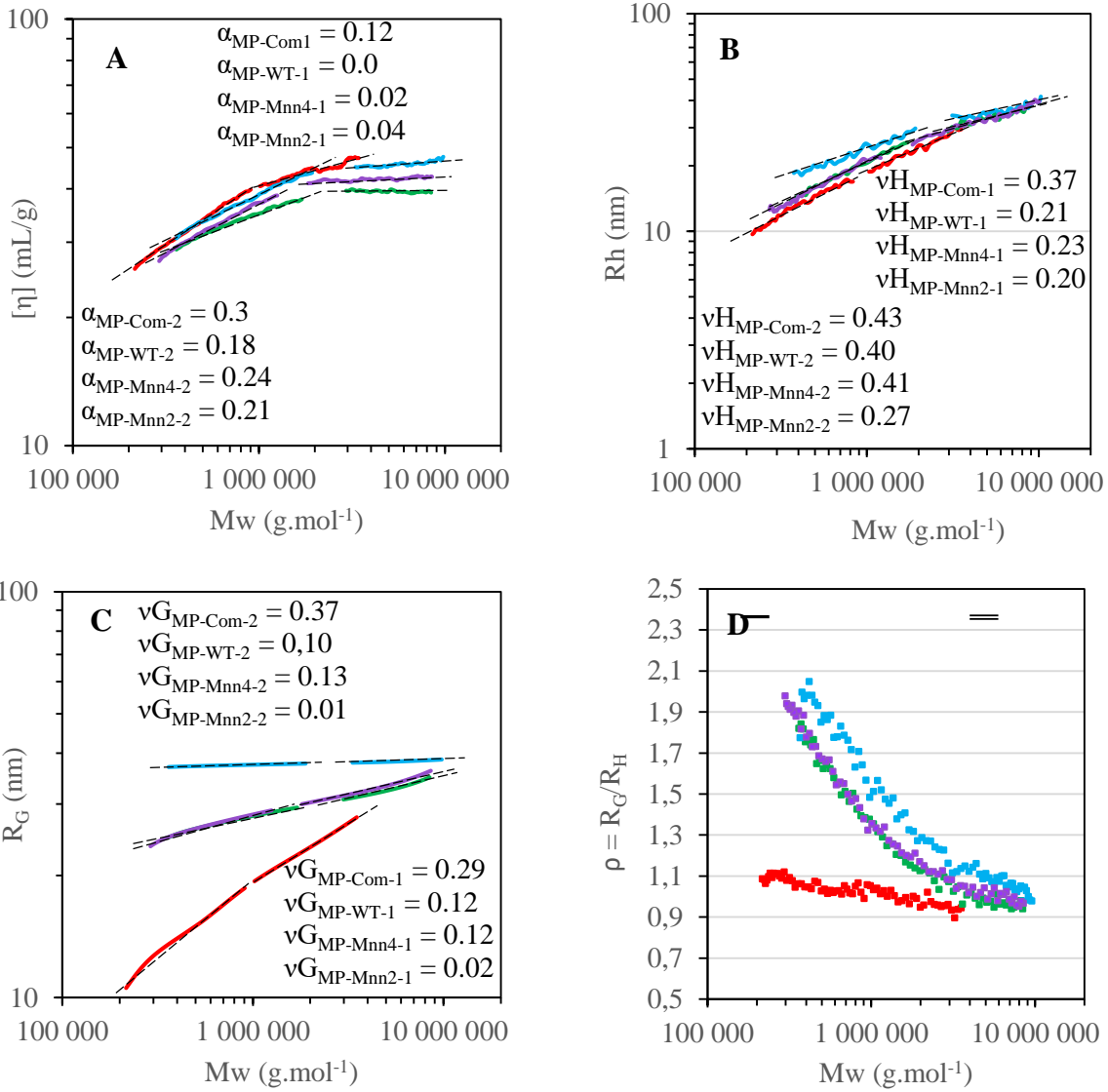
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**Figure 4 :** HPSEC-MALS profiles of the four pools of mannoproteins. A) MP-Com (Red), B) MP-WT (Green), C) MP-Mnn4 (Violet), and D) MP-Mnn2 (Light Blue). UV and RI detection profiles are presented on a relative scale. In the secondary axis, three molar mass profiles are presented for each mannoprotein: total molar mass (black lines); molar mass of the protein moiety (colored dotted lines); molar mass of the polysaccharides moiety (colored dashed lines).



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**Figure 5:** A) Mark-Houwink-Sakurada (MHS) plot showing the Intrinsic Viscosity as a function of the molar mass ( $M_w$ ) and the  $\alpha$  coefficients ( $[\eta] = K_\alpha M_w^\alpha$ ) for the 2 main populations (1 and 2) of each MP. B) Radius of gyration ( $R_g$ ) as a function of the molar mass ( $M_w$ ) and  $\nu G$  coefficients ( $R_g = K_g M_w^{\nu G}$ ) for the 2 main populations (1 and 2) of each MP. C) Hydrodynamic Radius ( $R_h$ ) as a function of the molar mass ( $M_w$ ) for the main fractions of each pool of MPs and the average  $\nu H$  coefficient ( $R_h = K_h M_w^{\nu H}$ ). D) The structural parameter  $\rho$  ( $\rho = R_g/R_h$ ) as a function of the molar mass ( $M_w$ ) for the two main fractions of the four pools of mannoproteins. MP-Com (Red); MP-WT (Green); MP-Mnn4 (Violet) and MP-Mnn2 (Light Blue).

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The Intrinsic Viscosity  $[\eta]$ , radius of gyration ( $R_g$ ), hydrodynamic radius ( $R_h$ ), and  $\rho$  parameter ( $\rho = R_g/R_h$ ) also known as asymmetry or anisotropy parameter) are plotted as a function of the molar mass  $M_w$  in **Figures 5A, 5B, 5C, and 5D**, respectively. For monodisperse polymers of sufficiently high molar mass and at infinite dilution,  $[\eta]$ , ( $R_g$ ), and  $R_h$  are related to the  $M_w$  through a simple power law:

$$[\eta] = K_\alpha M_w^\alpha \quad (1)$$

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$$R_g = K_g M_w^{\nu G} \quad (2)$$

490 
$$R_h = K_h M_w^{\nu H} \quad (3)$$

491 where  $\alpha$ ,  $\nu G$ , and  $\nu H$  are the corresponding hydrodynamic coefficients and  $K_\alpha$ ,  $K_g$ , and  $K_h$  the  
492 corresponding constants. The values of  $\alpha$ ,  $\nu G$ , and  $\nu H$  provide some insights into the shape,  
493 anisotropy, and solvation of the macromolecules in the medium (good solvent or not)  
494 (Burchard, 1999). The  $\rho$  parameter is dependent on the polymer architecture and conformation  
495 (**Supplementary data, Table 2**). Since the polymer flexibility, polydispersity, density, and  
496 homogeneity affect directly  $R_g$ , they also influence the  $\rho$  values.

497 The Mark-Howink-Sakurada plots ( $[\eta] = f(M_w)$ ) of the four mannoprotein pools  
498 evidenced a change in slope from population 2 to population 1 (**Figure 5A**). The impossibility  
499 to resume the whole curve in one power law (and consequently, one  $\alpha$  coefficient) highlighted  
500 structural differences between these two populations (Ioan et al., 2000; Lopez-Torrez, Nigen,  
501 Williams, Doco, & Sanchez, 2015).  $\alpha$  values go from 0 (corresponding to a sphere-shape  
502 population) to 1.8 (rod-like shape). Flexible polymers usually have intermediate  $\alpha$  values (0.5-  
503 0.8), dependent on the interactions between the macromolecules and the solvent (Burchard,  
504 1999).  $\alpha$  evolves inversely to their degree of branching and values below 0.5 indicate a  
505 hyperbranched polymer structure. Also,  $\alpha$  decreases as the  $M_w$  increases for macromolecules  
506 with a branching degree higher than 0.5. The  $\alpha$  values of population 2 in the four pools varied  
507 between 0.17 and 0.3. Such coefficients correspond to hyperbranched polymers with a  
508 homogeneous repartition through the hydrodynamic volume and suggest that most of the  
509 volume of the macromolecules remain well solvated and loosen, yet with an undefined shape.  
510  $\alpha$  values below 0.5 were also found upon a wide range of molecular weight for Acacia Seyal  
511 and Senegal Gums (Apolinar-Valiente et al., 2019; Lopez-Torrez et al., 2015), which are other  
512 natural hyperbranched polysaccharides. A clear decrease in  $\alpha$  was observed between population  
513 2 and population 1 for all MPs, the  $[\eta]$  becoming independent on  $M_w$  ( $\alpha \sim 0$ ) for MP-WT,

514 Mnn4, and Mnn2. This decrease suggests a difference in conformation and an increase in  
515 density between the two populations due to an increase in the branching degree and/or length  
516 of the chains (Ioan, Aberle, & Burchard, 1999; Rolland-Sabaté, Colonna, Mendez-Montevalvo,  
517 & Planchot, 2007; Rolland-Sabaté et al., 2008). Ioan et al. (1999) also observed  $\alpha$  values close  
518 to zero with glycogens, which are natural hyperbranched polysaccharides (Ioan et al., 1999).  
519 Although the branching degree of its polysaccharide moiety was much smaller (**Table 4**),  $\alpha$   
520 values found for both MP-Mnn2 populations were similar to those determined for MP-WT and  
521 MP-Mnn4. Differences in branching degree calculated by glycosyl-linkage analysis between  
522 these mannoproteins are related to the N-glycosylated chains. They do not reflect the degree of  
523 branching of the protein part by O and N-glycan carbohydrate chains. These similar  $\alpha$  values  
524 suggest that the molar mass dependence of  $[\eta]$  is mainly governed by the degree of branching  
525 of the protein moiety.

526 For the same  $M_w$ , differences were observed in the  $R_g$  and  $R_h$  values of the  
527 mannoproteins (**Figure 5B and C**). Both the  $\nu G$  (around 0.3) values obtained for the two  
528 populations of MP-Com (Red) were consistent with a sphere-like conformation (Ioan, 1999).  
529 The  $\nu H$  values around 0.4 found on the whole  $M_w$  distribution (between the sphere and the  
530 random coil theoretical values) indicated some anisotropy of the hydrodynamic volumes of the  
531 macromolecules (**Figures 5B and C**). The  $\nu H$  values determined within the “low”  $M_w$  range  
532 for MP-WT and MP-Mnn4 were closed to that found for MP-Com (~ 0.4), whereas it was lower  
533 for MP-Mnn2 and close to the theoretical one for a sphere shape (~ 0.3). Contrarily to what was  
534 observed with MP-Com,  $\nu H$  values decreased between the lowest and the highest molar mass  
535 populations, by a factor of 2 for MP-WT and Mnn4 and to a lower extent for MP-Mnn2. On the  
536 other hand, the  $\nu G$  coefficients determined for the two populations of the WT, Mnn2, and Mnn4  
537 mannoproteins were much lower than those found for MP-Com: they varied between 0.1 and  
538 0.13 for the MP-WT and MP-Mnn4 and dropped to values between 0.012 and 0.017 for the

539 MP-Mnn2. These quite low values indicated a particular behavior in terms of molecular mass  
540 dependence of the radius of gyration and were much lower than those usually reported for other  
541 branched/hyperbranched polysaccharides (between 0.27 and 0.5) (Lopez-Torrez et al., 2015;  
542 Rolland-Sabaté et al., 2007, 2008). They were also lower than that theoretically expected for  
543 spherical particles of uniform density ( $\nu G = 1/3$ ). Mannoproteins are heteropolymers consisting  
544 of a proteic moiety that carries polysaccharide side chains. They may differ within the same  
545 population in terms of the number, size, and structure of these carbohydrate chains (O and N-  
546 glycosylated chains), as well as in the structure of the later. These macromolecules are thus  
547 hardly comparable to literature data established with synthetic polymers with well-defined  
548 compositions and architectures or even with other branched polysaccharides. They can be  
549 explained if the increase in  $M_w$  throughout the distribution is associated with an increase in the  
550 branching density by carbohydrate side chains (number of branching points) and/or chain length  
551 (Burchard, 1999; Ioan et al., 2000; Rolland-Sabaté et al., 2008). Studying synthetic copolymers  
552 containing different and controlled amounts of long-chain branching, Auhl et al. observed a  
553 deviation from the power law that increased with the molar mass, with  $R_g$  values that reached  
554 a plateau ( $R_g$  independent of  $M_w$ ) at high molar masses, behavior that clearly indicated long  
555 chain branching in this specific case (Auhl et al., 2004). A similar dependence was found for  
556 statistically branched homopolymers with a branching content increasing with molar mass  
557 (Gabriel & Münstedt, 2002; Jackson, Chen, & Mays, 1996) and for dextrans (Ioan et al., 2000).  
558 Such an increase in branching density between the low and high molar mass populations for  
559 MP-WT, Mnn4, and Mnn2, resulting in an increase in molecular density, is consistent with the  
560 changes observed in  $\alpha$  values between the two populations of these mannoproteins and with the  
561 decrease in  $\nu H$ : when associated with an increase in branching, the increase in  $M_w$  does not  
562 induce the strong changes in  $R_h$  that would be observed for less branched polymers.

563 The  $\rho$  parameter found for MP-Com and the high molar mass population in the three  
564 other MP pools varied with the  $M_w$  between 1.1 and 0.9, which is consistent with dendrimeric  
565 (0.977) or regular star (1.079-1.33) structures (**Supplementary Data, Table 2**), and is within  
566 the range of  $\rho$  found for other branched polysaccharides such as amyloses, dextrans, glycogens,  
567 and some fractions of Acacia Senegal and Acacia Seyal gums (Ioan et al., 1999; D Renard et  
568 al., 2014; Rolland-Sabaté et al., 2008). By contrast, for population 2 of MP-WT/Mnn4/Mnn2,  
569 a strong decrease of  $\rho$  from 2 to 1.1 was observed when the  $M_w$  increased. Theoretical  $\rho$ -values  
570 above 1.3 are usually related to linear random coil structures, which do not correspond to  
571 mannoproteins as confirmed by the power law coefficients obtained from MHS,  $R_g \times M_w$ , and  
572  $R_h \times M_w$  plots and the glycosyl-linkage composition analysis. In this specific case, this is related  
573 to the molar mass dependence of  $R_g$  and  $R_h$ , attributed to an increase in branching density by  
574 carbohydrate side chains with the  $M_w$  and/or increasing side chain length.

575 Overall, these results clearly show structural differences between MP-Com, MP-  
576 WT/Mnn4, and MP Mnn2, and differences between the “low” and “high” molar mass  
577 populations within the four pools. Any comparison with other biopolymers was limited by the  
578 fact that there is no consistent literature data on  $\alpha$ ,  $v_G$  and  $v_H$  coefficients of mannoproteins,  
579 and that their composition as a natural copolymer is quite specific.

#### 580 **4 Conclusion**

581 The extraction and purification methods applied in this study made it possible to obtain  
582 pools of mannoproteins from 4 yeast strains: a laboratory wild-type strain (BY4742), two of its  
583 mutants ( $\Delta Mnn4$  and  $\Delta Mnn2$ , in which genetic deletions affect the polysaccharide moiety), and  
584 a commercial enological strain (LMD47) as control. The differences in the molecular  
585 characteristics of mannoproteins caused by genetic deletions ( $\Delta Mnn2$  and  $\Delta Mnn4$ ) on the strain  
586 BY4742 were confirmed and differences were also observed between the laboratory and the  
587 enological strains.

588           The composition of the protein and polysaccharide moieties of MPs showed that the protein  
589 moiety between the four MPs pools are similar and that the main difference comes from the  
590 polysaccharide part. As expected, differences from the normal polysaccharide structure of MP-  
591 Mnn2 and MP-Mnn4 when compared to MP-WT altered their net charge balance – usually  
592 ruled by both the protein and polysaccharide moieties – and the molecular conformation of MP-  
593 Mnn2. These characterizations also evidenced differences in structure, composition, and  
594 properties between MP-Com and MP-WT. Moreover, the comparison of the mannoproteins  
595 obtained in this work with the literature data, in terms of net charge, apparent and/or real  
596 molecular weight distributions, and protein content confirmed that the extraction method  
597 developed has little effect on the native structure of mannoproteins. In fact, mannoproteins  
598 purified from wine or extracted by the use of enzymatic cocktails (such as Zymolase® or  
599 Glucanex®) are also submitted to proteolytic and/or mannolytic activities and might not be  
600 representative of their structure in the yeast cell wall. The analysis of the static and dynamic  
601 parameters provided by HPSEC-MALLS revealed very specific conformational properties of  
602 mannoproteins related to their nature of highly branched natural copolymers with two branching  
603 levels: a protein more or less branched by irregular polysaccharides chains, which in turn are  
604 also more or less branched. However, any comparison with other sources of mannoproteins was  
605 limited by the fact that there is no literature data on  $\alpha$ ,  $v_G$  and  $v_H$  coefficients for these  
606 macromolecules.

607           The four mannoprotein pools obtained and characterized will be used to study how the  
608 structure of the polysaccharide moiety, which affects the structure and conformation of the  
609 macromolecule, and mannosyl-phosphate groups, which carry a negative charge at wine pH,  
610 impact their interactions with wine constituents and their functional properties in enology.



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