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Effect of the presence of lysated lees on polysaccharides, color and main phenolic compounds of red wine during barrel ageing

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ABSTRACT

A practice in wineries is to age wine in presence of lysated lees instead of fresh lees, in order to reduce the time wine is conserved on lees and avoid possible microbiological and organoleptic risks caused by lees. Two treatments were used to induce lees lysis: acidification and acidification in combination with a mixture of β-glucanases and pectinases. Acidification treatment in combination with enzymes induced significantly greater mannoprotein and glucan release. The presence of lysated lees during wine storage in barrels produced wines with significantly different tannin contents. The ageing technique on lysated lees by acidification in combination with enzymes produced wines with more intense colors, lower luminosity and saturation, and a slight tendency towards red tones, and an increase in sweetness, fullness and mouth length. On the contrary, ageing technique on lysated lees by acidification increased wine acid and fresh sensations.

Key words: lees, induced lysis, acidification, β-glucanases/pectinases, proanthocyanidins, monoglucosylated anthocyanins, color and sensorial analysis.

1 ***INTRODUCTION***

2 Today, many wineries age red wines on lees in order to obtain higher quality wines with
3 better structures, aromatic profiles and color stability. However, this technique requires
4 substantial investment in resources (vats, barrels, labor, sensorial analyses and
5 *batonnâges*) and is not free of problems, disagreeable scents of reduction and risk of
6 microbial deviation (Palomero, Morata, Benito, Calderón & Suárez-Lepe, 2009;
7 Rodríguez, Lezaún, Canals, Llaudy, Canals & Zamora, 2005).
8 When wine is kept in contact with lees, the yeast covering is naturally and slowly
9 degraded when must nutrient supplies are depleted. This microbiological phenomenon,
10 known as autolysis, is mainly induced through different enzymatic activities of the yeast
11 itself. This degradation in wine enriches products from different cell parts (Mazauric &
12 Salmon, 2005); the polysaccharide fraction released has one of the strongest effects on
13 the sensorial and physical-chemical properties of wines aged on lees (Palomero, Morata,
14 Benito, Calderón, & Suárez-Lepe, 2009). Thus, released mannoproteins limit tartaric
15 and protein precipitation in wines (Waters, Pellerin & Brillouet, 1994; Moine-Ledoux &
16 Dubourdieu, 2002) hence, their presence could prevent drastic stabilisation treatments
17 that impoverish wine (Rodríguez, Lezaún, Canals, Llaudy, Canals & Zamora, 2005).
18 Their positive effect on the organoleptic quality of wines has already been described
19 insofar as they modify gustatory structure, fullness and body (Vidal et al., 2004) and
20 soften tannin astringency (Riou, Vernhet, Doco & Moutounet, 2002; Feuillat, Escot,
21 Charpentier & Dulau, 2001). Due mainly to these properties, oenologists try to increase
22 the mannoprotein content of red wine, either by fermentation using strains of
23 mannoprotein overproducing yeast strains (Guadalupe, Palacios & Ayestarán, 2007;
24 Guadalupe, Martínez & Ayestarán, 2010), or during ageing using natural fresh lees or

25 commercial mannoprotein-rich preparations (autolysates, industrial yeast derivatives or
26 extracts).
27 Many years ago, the winemaking industry developed commercial enzyme preparations
28 in order to accelerate autolysis in wines aged on lees. These products are mixes of
29 several enzymes, such as β-glucanases and pectinases, which considerably increase
30 polysaccharide concentration in both white and red wines (Pellerin & Tessarolo, 2001;
31 Trione & Martínez, 2001). However, adding these enzymes directly to the wine in the
32 presence of lees prompts a significant increase in glucose, which, as a source of carbon,
33 may stimulate the growth of undesirable microorganisms such as *Brettanomyces*
34 (Guilloux-Benatier & Chassagne, 2001).
35 Based on these considerations, it is not surprising that oenologists continue to prepare
36 lysates from fresh lees produced in wineries. To do so, they accelerate the lysis of lees
37 previously employed in wine ageing using plasmolytic and hydrolytic agents
38 (Fornairon-Bonnefond, Camarasa, Moutounet & Salmon, 2002), such as commercial
39 tartaric acid or β-glucanases. This yields lysates lees rich in parietal polysaccharides and
40 products from different cell parts. Additionally, potential microbiological risks and
41 unpleasant reduction odors only affect the small volume of wine around the fine lees
42 and not all the wine aged on lees. However, the effect of these lysates lees produced in
43 wineries on wine composition and organoleptic quality is still not well understood.
44 This study examines the effect of the presence of lysates lees produced in wineries on
45 polysaccharides, color and the main polyphenolic compounds of red wine, as well as on
46 the organoleptic quality of wine during barrel ageing. The lysates less were prepared by:
47 i) acidification; and ii) acidification in combination with commercial β-glucanases.

48 ## *MATERIAL AND METHODS*

49 **Reagents**

50 All the reagents used were of analytical quality. All the chromatographic solvents were
51 of HPLC quality. L-fucose, L-rhamnose, 2-*O*-methyl-D-xylose, L-arabinose, D-xylose,
52 D-galactose, D-glucose, D-mannose, Kdo and vanillin were provided by Sigma (St.
53 Louis, MO), and D-galacturonic and D-glucoronic acid, myo-inositol by Fluka (Sigma).
54 D-apiose was obtained from Omicrom (South Bend, IN) and malvidin-3-glucoside and
55 peonidin-3-glucoside were provided by Extrasynthèse (Lyon, France).

56 Ethanol 96% (v/v), hexane and acetyl chloride were supplied by Scharlab (Barcelona,
57 Spain), hydrochloric acid 37% was purchased from Carlo Erba (Rodano, Milan, Italy),
58 and dried methanol, pyridine, hexamethyldisilazane and trimethylclorosilane were
59 obtained by Merck (Darmstadt, Germany). Vanillin, formic acid, acetonitrile,
60 trifluoroacetic acid and acetone were obtained from Sigma (St. Louis, MO).
61 Ammonium formiate of HPLC grade supplied by Fluka (Buch, Switzerland) and MilliQ
62 deionized water (Millipore, Molsheim, France) were used. A pullulan calibration kit
63 (Shodex P-82) was obtained from Waters (Barcelona, Spain). All the solutions were
64 filtered through a 0.45 μm filter before use in the HPLC.

65 The enzymes used (β-glucanases and pectinases) were supplied by Novozymes
66 Biopharma (Theberton, Australia).

67 **Obtainment of wine and lysated lees**

68 This study was carried out with wine produced at the CVNE winery (D.O.Ca Rioja).
69 The wine was made from Tempranillo grapes using vinification techniques designed to
70 obtain high concentrations of anthocyanins and proanthocyanidins. Thus, in the pre-
71 fermentative stage, 4% of must was removed to increase the solid/liquid ratio and a long
72 maceration period was used (21 days), with daily cap punching down and pumping

73 over. After malolactic fermentation, the wine was racked and sulphited with 30 mg/l of
74 SO_2. Alcoholic grade (14.5% v/v), pH (3.78), titratable acidity (6.23 g of tartaric acid
75 per liter), volatile acidity (0.54 g of acetic acid per liter), malic (0 g of malic acid per
76 liter) and lactic acid values (1.93 g of lactic acid per liter) indicated that the wine was
77 suitable for ageing and was therefore used in the experiment.
78 After racking of the red wines after malolactic fermentation, the lees deposited on the
79 bottom of the vat were recovered in a proportion of 80:20 (v/v) lees and wine, and used
80 for the experiment. The collected lees were mixed in two vats (L1 and L2), where they
81 were treated with tartaric acid (2.5 g/l) and sulphurous acid to 40 mg/l of free SO_2; this
82 treatment was referred to as the control (LC). Then, the lees were distributed in twelve
83 American oak used barrels (*Quercus alba*, fine grain, medium toasting, ten years used):
84 six were filled with acidified lees from vat L1 (L1C) and the other six were filled with
85 acidified lees from vat L2 (L2C). Then, 15 g/Hl of a commercial mixture of pectinases
86 and β-glucanases were added to half the barrels with L1C and L2C. The acidified lees
87 combined with the enzymatic treatment were labeled L1CE and L2CE. All the barrels
88 were rotated daily and kept at a temperature of 10ºC. The free sulphurous acid was
89 analyzed regularly and kept at between 35 and 40 mg/l.
90 The first lees sample was taken at the beginning of the lysis process ($L1C_{0\ days}$ and $L2C_{0}$
91 $_{days}$) and then samples were taken after 21 days ($L1C_{21\ days}$, $L1CE_{21\ days}$, $L2C_{21\ days}$,
92 $L2CE_{21\ days}$) and 60 days ($L1C_{60\ days}$, $L1CE_{60\ days}$, $L2C_{60\ days}$, $L2CE_{60\ days}$). After 60 days,
93 the lysated lees $L1C_{60\ days}$ and $L2C_{60\ days}$ were mixed and the resulting mixture was
94 called LC. The same operation was performed with lees $L1CE_{60\ days}$ and $L2CE_{60\ days}$ and
95 the resulting mixture was called LCE. The lysated lees LC and LCE were recovered in a
96 proportion of 80:20 (v/v) lees and wine, and microscopic inspection and counting in a
97 Neubauer chamber revealed a population of 3 x 10^8 lysed cells/ml.

98 **Ageing of the red wine on presence of lysated lees in oak barrels**

99 The wine selected for the experiment and described previously was distributed in nine
100 new 225-litre French oak barrels (*Quercus petraea*, medium grain, medium toasting).
101 Three of the nine barrels were treated as controls (C) and 9 liters of lysated lees using
102 the combined acidification and enzyme treatment (LCE) were added to another three
103 barrels and 9 liters of acidified lees (LC) were added to the remaining barrels. The
104 ageing period in the presence of lysated lees was three months, while the wines in
105 barrels without lysated were aged for a further six months.

106 Wine samples were taken for subsequent analysis during ageing in the presence of
107 lysated; the first sample was taken when lysated was added (t =0), the next sample was
108 taken after 45 days (t =45), and the final sample after 90 days (t =90). A sample of the
109 control wine (C) was taken after nine months of ageing.

110 **Determination of general enological parameters**

111 Conventional enological wine parameters (alcoholic grade, pH, titratable acidity, free
112 sulphurous acid and volatile acidity) and the chromatic characteristics at wine pH were
113 determined in accordance with official OIV methods (Office International de la Vigne et
114 du Vin, 1990).

115 **Obtainment of polysaccharides**

116 Polysaccharides were obtained from the samples by precipitation with ethanol-acid. The
117 lees and wine samples were homogenized and 50 ml were collected using a peristaltic
118 pump and centrifuged (9500 x g, 20 min, 4ºC). Polysaccharides were precipitated by
119 addition of four volumes cold acidified ethanol to the supernatants (ethanol of 96%
120 containing HCl 0.3 M) and kept for 18h at 4 ºC. Then, the samples were centrifuged, the
121 supernatants discarded, and the pellets washed several times with 96% ethanol to
122 remove the interference materials. The precipitates were finally dissolved in ultrapure

123 water and freeze-dried using a Virtis freeze drying (New York, USA). The lyophilised
124 fractions obtained (S fractions) contained the total soluble polysaccharides. Each
125 fraction was obtained in triplicate. The glucidic composition of the S fractions was
126 determined by gas chromatography, as described below.

127 **Polysaccharide identification and quantification by GC-FID**

128 The monosaccharide composition of the S fractions was determined by gas
129 chromatography with flame ionization detector (GC-FID) of their trimethylsilyl-ester O-
130 methyl glycosyl residues obtained after acidic methanolysis and derivatization. GC was
131 performed with a Hewlett-Packard HP5890 gas chromatograph (Hewlett-Packard, USA)
132 using a fused-silica capillary column (30 m x 0.25 mm x 0.25 µm, Teknokroma,
133 Barcelona, Spain) with helium as carrier gas and the rest of conditions previously
134 described (Ayestarán, Guadalupe & León, 2004). Each analysis was carried out in
135 triplicate. Total polysaccharides were quantified by adding all the analyzed sugars,
136 whereas the acidic and neutral sugars were obtained from neutral (apiose, arabinose,
137 rhamnose, fucose, xylose, mannose, galactose, 2-*O*-methyl-fucose, 2-*O*-methyl-xylose)
138 and acidic residues (aceric, galacturonic, glucuronic and 3-deoxy-octulosonic acid)
139 respectively. Polysaccharides rich in arabinose and galactose (PRAGs) and
140 rhamnogalacturonan II (RG-II) contents were estimated from the concentrations of their
141 characteristic monosaccharides (Ayestarán, Guadalupe & León, 2004; Doco, Quellec,
142 Moutounet & Pellerin, 1999).

143 **Distribution of polysaccharide molecular weights by HRSEC-RID**

144 To determine the molecular distribution of the polysaccharides obtained, the S fractions
145 were analyzed by high high-resolution size-exclusion chromatography (HRSEC) using
146 two Shodex OHpack KB-803 and KB-805 columns (30 x 0.8 cm, Showa Denko, Japan)
147 equilibrated with 1 ml/min of $LiNO_3$ 0.1M. Chromatographic separation was carried out

148 at room temperature on an Agilent modular 1100 liquid chromatograph (Waldbronn,
149 Germany) connected to a G1362 refractive index detector. The molecular weight
150 distribution of the lees and wine fractions was followed by calibration with narrow
151 pullulan molecular weight standards (Shodex P-82, Waters, Barcelona, Spain): P-5, Mw
152 = 5.9 KDa; P-10, Mw = 11.8 KDa; P-20, Mw = 22.8 KDa; P-50, Mw = 47.3 KD; P-100,
153 Mw = 112 KDa; P-200, Mw = 212 KDa; P-400, Mw = 404 KDa. The apparent
154 molecular weights were deduced from the calibration equation log M_w = 8.182 – 0.403
155 t_R (t_R = column retention time at peak maximum, and r^2 = 0.999). Each analysis was
156 carried out in triplicate.

157 **Determination of color parameters**

158 Spectrophotometric measurements were taken in a 300 Scan Cary UV-Vis
159 spectrophotometer (Inc, Madrid, Spain Vary) using quartz cells with path lengths of 1-
160 mm, 2-mm and 10-mm. All absorbance values were corrected to 10-mm of length. Each
161 measurement was performed in triplicate.

162 The CIELAB parameters, luminosity (L *), chrome (C *) and tone (H *) were
163 determined in accordance with Ayala, Echávarri & Negueruela, (1997).

164 **Determination of anthocyanins by HPLC-DAD**

165 High-resolution size-exclusion chromatography with diode array detection (HPLC-
166 DAD) was performed in an Agilent 1100 modular liquid chromatograph (Waldbronn,
167 Germany) equipped with a G1313A injector, a G1311A HPLC quaternary pump, an on-
168 line G1379A degasser, a G1316A oven, a G1315B photodiode array detector and
169 Agilent Chemstation software. The column was a reversed-phase Kromasil 100-C18 (5
170 µm packing, 200 x 4.6 mm i.d.) protected with a guard column of the same material
171 (Teknokroma, Barcelona, Spain). The anthocyanins were eluted under the following
172 conditions: 1 ml/l flow rate; oven, 30ºC; solvent A: formic acid/water (2:98, v/v);

173 solvent B: acetonitrile/water/formic acid (80:18:2, v / v / v); gradients: isocratic 2% B in
174 10 min, from 2 to 10% B in 2 min, from 10 to 15% B in 10 min, from 15 to 30% B in 10
175 min, from 30 to 50% B in 20 min, from 50 to 60% B in 5 min. Wine samples were
176 directly chromatographed (50 μL). UV-visible spectra were recorded every second from
177 250 nm to 600 nm, with a bandwidth of 1.2 nm, and the chromatograms were acquired
178 at 515 nm for anthocyanins. The different compounds were identified according to their
179 UV-visible spectra, retention times and by comparison with commercial standards. The
180 calibration curves were obtained by injecting different concentrations of malvidin-3-
181 glucoside standards. The range of the linear calibration curves (r^2>0.99 in all cases) was
182 from 0.01 (detection limit) to 1 mg l^{-1} for the lower concentration compounds and from
183 1.0 to 100 mg l^{-1} for the higher concentration compounds. Each measurement was run in
184 triplicate.

185 The non-acylated anthocyanins (A-Glu) were calculated as the sum of delfinidin,
186 cyanidin, petunidin, peonidin and malvidin-3-glucosides; the acetylated anthocyanins
187 (A-Ac) as the sum of delfinidin, cyanidin, petunidin and malvidin-3-(6-acetyl)-
188 glucosides; and the cumarylated anthocyanins (A-Cm) as the sum of delfinidin,
189 petunidin and malvidin-3-(6-*p*-cumaryl)-glucosides. Total anthocyanins (T-A) were
190 calculated as the sum of A-Glu, A-Ac and A-Cm.

191 **Determination of tannin content**

192 Tannin content was determined using the vanillin method described by Sun, Ricardo da
193 Silva & Spranger, (1998). In order to avoid interferences caused by the monomeric
194 anthocyanins of the wine, the wine samples were previously fractioned by gel
195 permeation chromatography (GPC) on a Toyopearl gel HP-50F (Tosohaas,
196 Montgomery-ville, PA, USA) as described by Vidal et al., (2004). The first fraction
197 (F1) was eluted with ethanol/water/trifluoroacetic acid (55:45:0.05, v/v/v) and was

198 mainly composed of monomeric phenols (flavan-3ols, anthocyanins, flavonols and
199 phenolic acids) (Vidal et al. 2004; Sun, Ricardo da Silva & Spranger, 1998) and the
200 second (F2) was recovered by elution with acetone/water (60:40, v/v). Fraction F2 was
201 vacuum dried and total tannin content (Tan) was determined in the fraction.

202 **Sensory Analysis**

203 Aged wines were analyzed by a panel of expert tasters for sensory profiling. Wines
204 were judged on visual (color), olfactory (volatile fraction) and gustatory (taste and
205 mouth-feel sensations). A panel of twelve tasters, wine professionals from the D.O.Ca.
206 Rioja, was convened. All wine tasters had participated on previous aroma and mouth-
207 feel sensory descriptive panels and had regularly participated in quality scoring
208 Tempranillo wine sensory panels. The wines were presented at 18 °C in coded standard
209 wine-tasting glasses according to standard 3591 (ISO 3591, 1997). Assessment took
210 place in a standard sensory analysis chamber (ISO 8589, 1998) equipped with separate
211 booths. Wines were presented in two stages. In a first session, the panelists were asked
212 to describe the gustative and olfactory attributes in their own words. Descriptive terms
213 and their definitions were debated among the assessors, and a common consensus
214 vocabulary was then compiled and discussed further with panelists. Tasters selected 10
215 attributes for the olfactory and gustative phase, which were agreed upon as best for
216 describing the sensory characteristics of the wines. All the generated terms were usual
217 wine terms for describing red wines. In following sessions, assessors used the consensus
218 vocabulary, scoring the intensity of each attribute on an interval scale with 6 levels of
219 intensity (0) no aroma or taste; 1) weak aroma or taste; 3) intense aroma or taste; 5)
220 extremely strong aroma or taste; intermediate values did not bear description. The color
221 was also judged, and blue-red color was rated according to its intensity on an anchored
222 scale with six levels of intensity (0) no blue-red color; 1) weak blue-red color; 3)

223 intense blue-red color; 5) extremely strong blue-red color. Wine samples were assessed
224 in triplicate.

225 **Statistical analysis**

226 The analyses were carried out in triplicate. The significant differences between the
227 samples were analyzed using the SPSS 15.0 program for Microsoft Windows (SPSS
228 Inc., Chicago, IL). Monosaccharide, polysaccharide, anthocyanin and tannin contents,
229 as well as color values and enological parameters, were analyzed by means of an two-
230 way analysis of variance (ANOVA), with repeated measures to check the effect of time,
231 when the data complied with normality characteristics. When this was not the case, non-
232 parametric tests were performed (Kruskal-Wallis test).

233 Sensory data were subjected to ANOVA using the SPSS 12.0 program to determine
234 reproducibility of attribute scores. Separate principal component analyses (PCA) were
235 carried out on the mean ratings for aroma and gustatory attributes. The PCA was
236 conducted using the covariance matrix with no rotation (XLSTAT 2007 program for
237 Microsoft Windows). Average configuration plot dimensions were interpreted taking
238 into account the descriptors used by each of the assessors, which were most highly
239 correlated with each dimension. In this study, there were no significant differences in
240 the scores given by the tasters for each attribute ($p<0.05$), indicating that all the
241 panelists used all attributes reproducibly.

242 ***RESULTS AND DISCUSSION***

243 **Evolution of the enological parameters of the wine containing treated lees**

244 Table 1 shows that titratable and volatile acidity values of the wine with the L1C lees at
245 the beginning of the lysis process were significantly different to those of the wine with
246 the L2C lees. These results were expected because the L1 and L2 lees were a mixture of
247 lees obtained from different wines.

248 The effectiveness of lees acidification was checked by measuring the pH and titratable
249 acidity of the wine containing the lees. This treatment reduced wine pH in 1.48±0.08
250 units and increased total acidity by 42.5%. As the treatment time of lees acidification
251 (LC) and acidification in combination with pectinases and β-glucanases (LCE)
252 increased, it was observed that pH values remained constant, while titratable acidity
253 increased significantly in the last month. When the two treatments (LC and LCE) were
254 compared on the last sampling date, it was observed that the wines containing lees had
255 similar pH and titratable acidity values.
256 As treatment time advanced, it was observed that the volatile acidity values of the wines
257 after 60 days were similar to those at the beginning of treatment. Volatile acidity ranged
258 from 0.2 to 0.6 g of acetic acid per liter, which is acceptable for any red wine that has
259 undergone alcoholic and malolactic fermentation (Guasch Torres, 2007). The volatile
260 acidity values indicated that the microorganisms present in the lees did not seriously
261 alter the wine containing them, probably due to the low pH conditions and high free
262 SO_2 dose used.

263 **Evolution of polysaccharides released into the wine during induced lysis of lees**

264 Figure 1 shows the distribution of the molecular weights of the polysaccharides released
265 in different lees samples. Five peaks eluted at approximately 15, 16.7, 18, 21.3 and 22.8
266 minutes. The first three peaks, which corresponded to molecules with molecular weights
267 between P-400 (404 kD) and P-5 (5.9 kD), were attributed to the presence of yeast
268 parietal polysaccharides and also mannans and mannoproteins, as well as other grape
269 polysaccharides such as polysaccharides rich in arabinose and galactose (PRAGs) and
270 rhamnogalacturonan II dimmers (Ayestarán, Guadalupe & León, 2004). The last two
271 peaks had lower molecular weights than P-5 and were due to the presence of low
272 molecular weight polysaccharides or other molecule fragments.

273 Figure 1A shows the distribution of the molecular weights of polysaccharides released
274 during lysis of the L2 lees treated by acidification (L2C). With time, the medium was
275 enriched with polysaccharides, mainly of high molecular weight. This was due to the
276 lysis of the lees, where parietal polysaccharides were released to the medium as
277 mannoproteins and glucans (Charpentier, Santos & Feuillat, 2004).
278 Figure 1B shows the distribution of the molecular weights of released polysaccharides
279 after 60 days of lysis by the L2 lees treated with acidification ($L2C_{60\ days}$) and by lees
280 acidified in combination with pectinases and β-glucanases ($L2CE_{60\ days}$). The Figure
281 shows that the profile of the polysaccharide was similar with both treatments.
282 Nevertheless, commercial enzyme treatment induced a greater response in the high
283 molecular weight peaks, indicating that the pectinase and β-glucanase enzymes induced
284 greater hydrolization of the high molecular weight polysaccharide fragments.
285 The content of total polysaccharides released during the lysis process in shown in Table
286 2. During the first 21 days of lysis, with both treatments total polysaccharide release
287 was substantially higher than the mean (68-116%), before declining until 60 days (12-
288 20%). When both treatments were compared on the final sampling date, it was observed
289 that the content of total released polysaccharides had increased by 40% for $L1CE_{60\ days}$
290 and 25% for $L2CE_{60\ days}$.
291 Among the sugars forming wine polysaccharides, neutral sugars represented 96±4% of
292 total sugars (Table 2). Of the neutral sugars constituting the polysaccharides released
293 mannose was the main monosaccharide, constituting 65±1% of the total in $L1CE_{60\ days}$
294 and $L2CE_{60\ days}$, and 56±5% of the total in $L1C_{60\ days}$ and $L2C_{60\ days}$. The next main
295 monosaccharide was glucose, which accounted for 17±2% of the total in L1CE and
296 L2CE and 9±1% of the total in L1C and L2C. Both sugars were the main components of
297 the microbial polysaccharides (Doco, Williams & Cheynier, 2007); their presence may

298 be used as an indicator of the quantity of mannoproteins and glucans in the medium
299 since the quantity of mannose is an estimate of the quantity of mannoproteins
300 (Ayestarán, Guadalupe & León, 2004), and the quantity of glucose is an estimate of the
301 quantity of glucans.
302 During lysis, large quantities of mannose and glucans were released into the medium, in
303 the acidified lees and the lees acidified with enzymes, although this was greater in the
304 lees with added commercial enzymes (Table 2). Thus, the addition of commercial
305 enzymes promoted an increase of 117±13 mg/l mannoproteins, a value 3.9 times greater
306 than that reported by Doco, Vuchot, Cheynier & Moutounet, (2003) in red wine after six
307 months of ageing on lees. The case of glucose was slightly different because only low
308 quantities (31±6 mg/l) of this sugar were released into the medium in the lees without
309 enzymatic treatment during the entire lysis process, in contrast to the lees treated with
310 enzymes in which glucose increased threefold (99±25 mg/l). This fact indicates that
311 simple acidification of the lees has a milder effect on glucans in lees cell walls and that
312 commercial enzymatic preparations must be added with β-glucanases to break these
313 polysaccharides. β-glucanase is known to hydrolyse β-glucan, which, together with
314 chitin, helps configure the cell-wall structure, forming a network supporting many
315 mannoproteins. Thus, in addition to inducing the transfer of polysaccharides and
316 mannoproteins to the medium, β-glucanase releases glucose and oligosaccharides when
317 the β-O-glycosidic bond assembling the β-glucan chains breaks (Palomero, Benito,
318 Morata, Tesfaye, González & Suárez-Lepe, 2009).
319 At the end of lysis, the addition of commercial enzymes to acidified lees prompted the
320 release of approximately 33% more mannose and 120% more glucose than in the lees
321 that were only acidified (Table 2). These differences were already perceptible after 21
322 days of lysis, indicating that induced lysis could decrease considerably in time.

323 **Effect of lysated lees on wine polysaccharides at the end of barrel ageing**

324 Table 3 shows the contents of total polysaccharides, acidic sugars and neutral sugars
325 and the different polysaccharide families after three months of barrel ageing of the wine
326 with the absence (control wine) and presence of lysated lees, as well as for acidification
327 (LC) and acidification in combination with pectinases and β-1,3 glucanases (LCE).
328 No significant differences were observed for total polysaccharide content in any of the
329 analyzed wines. Moreover, all the wines presented very similar neutral sugar
330 concentrations, which were always higher than concentrations of acidic sugars, which
331 represented 17% of total polysaccharides, a value similar to that reported by other
332 authors in Tempranillo wines (Guadalupe& Ayestarán, 2007). The concentrations of the
333 grape polysaccharides PRAGs and RG-IIs were similar in all the wines, representing
334 between 31% and 21% of total polysaccharides, respectively. Similarly, no significant
335 differences were observed between wines in terms of glucose and mannose content.
336 Clearly, the addition of 4% lysated lees to the wine was very low, but the normal dose
337 of lees used in wineries is 1-5%. Future studies will have to examine whether the dose
338 and/or degree lysis of the lees are factors that affect the enriching of mannoproteins in
339 wine during ageing.

340 **Effect of lysated lees on monomeric anthocyanins and tannins during ageing**

341 At the beginning of ageing, the addition of lysated lees did not induce significant
342 differences in the content of non-acylated anthocyanins (A-Glu), *p*-cumarylated
343 anthocyanins (A-Cm) and acetylated anthocyanins (A-Ac) in all the wines analyzed
344 (Table 4). The non-acetylated anthocyanins (A-Glu) represented 85% of total
345 anthocyanins, while the A-Cm derivatives represented 10% and the A-Ac 4.6% of the
346 total. As was expected, malvidin-3-glucoside was the majority anthocyanin,

347 representing 49% of total anthocyanins, and its derivatives were also the majority
348 among the *p*-cumarylated and acetylated derivatives.
349 After nine months of ageing of the control wine, A-Glu, A-Cm and A-Ac content
350 diminished; the rate was different for each group of monomeric anthocyanins studied
351 (data not shown). Normally, ageing of red wines in oak barrels entails the loss of
352 monomeric anthocyanins because these are degraded, turning into non-coloured forms
353 or polymerising into more stable forms (Palomero, Morata, Benito, González, &
354 Suárez-Lepe, 2007). This behavior is clearly associated with the presence of oxygen,
355 facilitated by the permeability of oak wood, enabling the formation of ethyl bridges
356 between anthocyanins and proanthocyanidins (Cheynier, Moutounet & Sarni-
357 Manchado, 2000). However, free anthocyanins tend to also disappear due to oxidation,
358 absorption and precipitation of flavanol polymer molecules (Rodríguez, Lezaún, Canals,
359 Llaudy, Canals& Zamora, 2005). However, during the first three months of ageing no
360 differences were observed in the evolution of the contents of non-acetylated and p-
361 cumarylated anthocyanins and acetylated derivatives in the wines (Table 4). The
362 concentrations of most of the different anthocyanin structures increased in the first 45
363 days and decreased in the final 45 days of ageing, although the concentrations of
364 majority anthocyanins (Mv-3-glc, Df-3-glc, Pt-3-glc, Mv-3-*p*cumglc, Mv-3-acetilglc)
365 after three months of ageing was similar to those at the beginning. The level of free SO_2
366 (~32 mg/l) in the wines at the beginning of ageing delayed the evolution of the
367 polyphenols during the first months of ageing.
368 The concentration of each anthocyanin structure studied in the last sample of wine aged
369 in contact with the lysates lees (t=90) did not display significant differences in the wines
370 analyzed (Table 4). These results made it impossible to differentiate the effect of adding
371 one type of lysate on the monomeric anthocyanin content of the wine from the effect

372 achieved with the other type of lysate and with the control. This result was due to the
373 experimental conditions used in this study: low quantity of lysate added to a wine
374 selected for its high anthocyanin concentration (Table 4). It is unlikely that either factor
375 favors anthocyanin loss by adsorption through the cell surface of the lysated yeasts.
376 Vasserot, Caillet & Maujean, (1997) observed in their tests that high initial anthocyanin
377 concentrations in the medium and low quantities of yeast lees reduce yeast wall
378 adsorption of anthocyanins.

379 The use of commercial β-glucanase enzymes in ageing on lees may result in total
380 anthocyanin loss probably via the undesirable activity of β-glucoside impurities
381 (Palomero, Morata, Benito, González, & Suárez-Lepe, 2007). However, this loss was
382 not observed with any of the lysates lees added. Consequently, the advantage of ageing
383 an anthocyanin-rich wine on a small quantity of lysated lees is that it does not lose
384 monomeric anthocyanins, which are, together with their derivatives, mainly responsible
385 for wine color.

386 Table 5 shows that at the start of ageing, tannin concentration was high and similar in
387 both the control wine and in the wine in the presence of acidified lysated (LC) and
388 acidified lysated in combination with pectinases and β-glucanases (LCE). It is known
389 that wood polyphenols are extracted by wine during ageing, which would explain that
390 tannin content in the third month of wine ageing was significantly higher than at the
391 beginning. Nevertheless, the increase in tannin content was greater in the control wine
392 (54%), followed some way behind by the LCE wine (27%) and the LC wine (20%).
393 Tannin content did not differ significantly in the wine aged on either LCE or LC, but its
394 content was significantly lower than that of the control. These results coincide with
395 those reported elsewhere (Rodríguez, Lezaún, Canals, Llaudy, Canals& Zamora, 2005),
396 which indicate that the disadvantage of ageing wines in the presence of lees is that it

397 produces wines with lower tannin content. One possible explanation for tannin loss is
398 their adsorption by the lysates lees; previous studies seem to indicate that tannins are
399 adsorbed on lees in preference to monomeric phenols, even with low quantities of lees
400 (Mazauric & Salmon, 2005). The observed effect of the lysates lees on the tannins was
401 similar to that described by our group for the commercial mannoproteins, which
402 reduced wine tannin content (Guadalupe, Palacios & Ayestarán, 2007).

403 **Effect of lysated lees on wine color during ageing**

404 Table 6 shows that the wine selected for ageing (t =0) has high ageing potential due to
405 its high color intensity value (CI) and absorbance at 280 nm and because the
406 anthocyanin and tannins concentrations were high (Tables 4 and 5).

407 As with the monomeric anthocyanins, the changes in most of the color parameters
408 during the ageing period were similar in the analyzed wines (Table 6). Thus, color
409 intensity increased slightly (between 1 and 2 units) due mainly to the slight increase in
410 red (A520nm) and yellow (A420nm) components. Hue increased slightly in the wines
411 analyzed, mainly after the first 45 days of ageing (from 0.05 to 0.07 units); hence, the
412 wines did not display a strong tendency to develop yellow tones during the first three
413 months of ageing. Nevertheless, CIE hue (H*) decreased significantly, as did luminosity
414 (L*) and chromaticity (C*).

415 The effect of the presence of the lysated lees on wine color after three months of ageing
416 seemed to depend on the type of treatment applied to the lees. Thus, L*, C* and H*
417 were significantly lower in the wine aged with lysated LCE, and this wine also had a
418 significantly greater color intensity (Table 6). These results indicated that the presence
419 of lysated lees in the acidification treatment in combination with enzymes produced
420 wines with greater color intensity and lower luminosity and saturation, as well as a
421 slight tendency to produce red tones. However, the effect of the presence of the

422 acidified lysated on color was insignificant because the parameters color intensity, L*,

423 C* and H* were significantly lower than those of the control wine.

424 **Effect of lysated lees on sensorial analysis during ageing**

425 Sensory evaluations of wines were performed after three months of aging in order to

426 verify the differences observed between wines on the organoleptic perception.

427 In the visual phase, wines did not show significant differences in their color intensities,

428 with mean punctuations close to 3.5. In the olfactory phase, no conclusive data were

429 obtained (data not shown). Figure 6 provide a graphic representation of the relationship

430 of the wines as determined by their gustatory perceptions. Wines were properly located

431 in the vectorial dimension defined by the first two factors, which accounted for 64.6%

432 of the total variance in the gustatory PCA space (Figure 6). Wines were properly

433 separated in the vectorial dimension defined by the first two factors; thus, the type of

434 treatment applied to the lees (LC or LCE) significantly affected to the gustative phase.

435 Control wines were highly related with the warm descriptor and wines in presence of

436 lysated by acidification (LC) had a strong effect on the mouth-feel, enhancing acid and

437 fresh sensations. On the other hand, acidification of lees in combination with pectinases

438 and β-1,3 glucanases increased sweetness, fullness and mouth length perceptions.

439 **Conclusions**

440 Acidification treatment of lees in combination with β-glucanases and pectinases

441 produced higher liberation of mannoproteins and glucans than acidification alone. The

442 practice of aging wine in presence of lysated lees had the advantage of not modifying

443 the content of monomeric anthocyanins but produced wines with lower tannin content.

444 After three months of wine aging on lysated lees, the only significant difference

445 between the use of lees obtained by acidification (LC) and lees obtained by acidification

446 in combination with enzymes (LCE) was observed in wine color parameters and

447 gustatory perceptions. Therefore, the presence of lysates LCE produced wines with
448 greater color intensity, as well as a slight tendency to produce red tones, and increased
449 sweetness, fullness and mouth length perceptions. On the contrary, the effect of the
450 presence of the acidified lysates (LC) on wine color was insignificant but it had a strong
451 effect on the mouth-feel, enhancing acid and fresh sensations.

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539

ILLUSTRATIONS

Figure 1. Distribution of the molecular weights of polysaccharides released during the lysis process lees through HRSEC with Shodex OHpack KB-803 and KB –805 columns. A) Evolution during lysis of lees treated by acidification (L2C). B) Differences between lysis induced by acidification ($L2C_{60\ days}$) and by acidification in combination with pectinases and β-glucanases ($L2CE_{60\ days}$).

Figure 2. Biplot of principal components I and II of the gustatory attributes of the wines after barrel aging. The three replicates of each wine are shown: Control (C1, C2 and C3), wine in the presence of acidified lees (LC1, LC2 and LC3), wine in presence of acidified lysate lees in combination with pectinases and β-glucanases (LCE1, LCE2 and LCE3).

Table 1. Evolution of the enological parameters of the wine containing lees during the lysis process. a) acidified lees (L1C and L2C); b) acidified lees + pectinases and β-glucanases (L1CE and L2CE)

	$L1C_{0\ days}$	$L1C_{21\ days}$	$L1C_{60\ days}$	$L1CE_{21\ days}$	$L1CE_{60\ days}$	$L2C_{0\ days}$	$L2C_{21\ days}$	$L2C_{60\ days}$	$L2CE_{21\ days}$	$L2CE_{60\ days}$
pH	3.67±0.1	2.20±0.04	2.27±0.03	2.22±0.06	2.3±0.08	3.82±0.1	2.21±0.09	2.26±0.03	2.26±0.07	2.37±0.07
Titratable acidity[a]	3.5±0.1	4.05±0.05	4.8±0.1	4.0±0.1	4.8±0.1	3.22±0.08	3.9±0.1	4.8±0.3	4.0±0.1	4.7±0.2
Volatile acidity[b]	0.48±0.1	0.5±0.1	0.6±0.2	0.51±0.08	0.6±0.2	0.45±0.04	0.46±0.08	0.54±0.09	0.48±0.07	0.52±0.06
Free SO_2[c]	45±1	38±2	40±2	39±2	36±2	45±2	40±2	37±2	39±2	36±2

[a] g of tartaric acid per liter
[b] g of acetic acid per liter
[c] expressed as mg/l

Table 2. Polysaccharides (mg/l) released during the lysis process. a) acidified lees (L1C and L2C); b) acidified lees + pectinases and β-glucanases (L1CE and L2CE).

	$L1C_{0\ days}$	$L1C_{21\ days}$	$L1C_{60\ days}$	$L1CE_{21\ days}$	$L1CE_{60\ days}$	$L2C_{0\ days}$	$L2C_{21\ days}$	$L2C_{60\ days}$	$L2CE_{21\ days}$	$L2CE_{60\ days}$
Total polysaccharides[a]	329±11	555±11	670±14	711±65	803±34	240±12	496±22	576±35	557±32	643±34
Neutral sugars[b]	320±11	543±10	661±14	702±65	795±34	228±11	477±22	560±34	546±32	633±33
Acidic sugar[c]	9.5±0.6	11±1	9.6±0.8	8.9±0.8	7.4±0.7	6.9±0.6	19±1	16±1	11±0.7	10.2±0.7
Mannose	148±4	299±24	402±31	468±44	528±50	118±5	238±19	303±11	332±31	410±32
Glucose	29.9±0.6	51±2	65±2	127±12	147±13	21±1	40±3	48±5	87±6	102±7

[a]: calculated as the sum of acidic and neutral sugars

[b]: calculated as the sum of apiose, arabinose, rhamnose, fucose, xylose, mannose, galactose, 2-*O*-methyl-fucose, 2-*O*-methyl-xylose

[c]: calculated as the sum of aceric, galacturonic, glucuronic and 3-deoxy-octulosonic acid;

Table 3. Content of polysaccharides and sugars of the wines at the end barrel aging. Wine control (Control), wine in the presence of acidified lysate lees (LC), and wine in the presence of acidified lysate lees in combination with pectinases and β-glucanases (LCE).

	Control	LC	LCE
Total polysaccharides (mg/l)[a]	272± 20[a]	281± 25[a]	271± 16[a]
Neutral sugars (mg/l)[b]	222± 18[a]	233± 22[a]	231± 4[a]
Acidic sugars (mg/l)[c]	47± 3[a]	48± 4[a]	46± 4[a]
RG-II (mg/l)	58± 4[a]	63± 6[a]	58± 2[a]
PRGAs (mg/l)	95± 4[a]	99± 9[a]	99± 9[a]
Mannose (mg/l)	77± 7[a]	90± 8[a]	83± 8[a]
Glucose (mg/l)	15.6±0 ,6[a]	15± 1[a]	16± 1[a]
Mannose+Glucose (mg/l)	93± 7[a]	106± 8[a]	99± 8[a]

[a]: calculated as the sum of acidic and neutral sugars

[b]: calculated as the sum of apiose, arabinose, rhamnose, fucose, xylose, mannose, galactose, 2-*O*-methyl-fucose, 2-*O*-methyl-xylose

[c]: calculated as the sum of aceric, galacturonic, glucuronic and 3-deoxy-octulosonic acid;

RG-II, rhamnogalacturonan-II; PRGAs, polysaccharides rich in arabinose and galactose

All data are expressed as the arithmetic mean ± standard deviation (n =9). Means in the same column followed by the same letter indicate the absence of significant differences ($p>0.05$).

Table 4. Content of the different monoglucosylated anthocyanins (mg/l) of the wines during barrel aging. Wine control (Control), wine in the presence of acidified lysate lees (LC), and wine in the presence of acidified lysate lees in combination with pectinases and β-glucanases (LCE).

	t = 0 days			t = 45 days			t = 90 days		
	Control	LC	LCE	Control	LC	LCE	Control	LC	LCE
Df-3-glc	46±2[a]	45±3[a]	47±3[a]	58±1[a]	59±2[a]	59±2[a]	51.4±0.5[a]	51±1[a]	53±3[a]
Ci-3-glc	3.2±0.2[a]	3.0±0.1[a]	3.14±0.07[a]	3.31±0.06[a]	3.8±0.3[a]	3.9±0.2[a]	3.79±0.03[a]	4.0±0.3[a]	3.6±0.2[a]
Pt-3-glc	42±2[a]	44±2[a]	42±1[a]	51.3±0.7[a]	52.0±0.4[a]	52±1[a]	44±2[a]	44±2[a]	45±4[a]
Pn-3-glc	13.6±0.5[a]	13.2±0.6[a]	13.6±0.2[a]	17±1[a]	15.2±0.1[a]	15.4±0.5[a]	16±1[a]	16±1[a]	15.02±0.08[a]
Mv-3-glc	142±5[a]	142±11[a]	142±3[a]	168±3[a]	167±2[a]	169±5[a]	145±2[a]	144±5[a]	147±3[a]
Df-3-acetiglc	3.7±0.2[a]	4.0±0.3[a]	3.9±0.1[a]	7.2±0.2[a]	4.7±0.2[b]	4.6±0.2[b]	5.0±0.3[a]	5.3±0.4[a]	5.3±0.2[a]
Ci-3-acetiglc	1.71±0.07[a]	0.9±0.02[b]	1.6±0.1[a]	6±2[a]	2.08±0.03[b]	2.2±0.2[b]	5.5±0.1[a]	5.41±0.08[a]	5.8±0.2[a]
Pt-3-acetiglc	1.88±0.09[a]	1.8±0.1[a]	1.86±0.05[a]	4.3±0.2[a]	2.25±0.04[b]	2.14±0.09[b]	5.5±0.3[a]	5.4±0.4[a]	5.6±0.5[a]
Mv-3-acetilglc	5.9±0.8[a]	5±1[a]	6.14±0.05[a]	9.3±0.5[a]	6.7±0.6[b]	6.7±0.5[b]	13±1[a]	12.46±0.08[a]	12.1±0.8[a]
Df-3-*p*cumglc	7.9±0.5[a]	7±0.4[a]	6.9±0.4[a]	12.9±0.3[a]	11.1±0.6[b]	10.5±0.4[b]	12.0±0.8[a]	11±1[a]	12.1±0.1[a]
Pt-3-*p*cumglc	3.8±0.2[a]	3.7±0.2[a]	3.7±0.2[a]	7.02±0.05[a]	5.60±0.04[b]	5.5±0.2[b]	5.9±0.3[a]	5.7±0.3[a]	5.9±0.2[a]
Mv-3-*p*cumglc	20±1[a]	21±1[a]	19.3±0.9[a]	26.0±0.6[a]	25.8±0.4[a]	26±1[a]	20.9±0.5[a]	20.4±0.6[a]	20.84±0.09[a]
A-Glc (mg/l)	247±6[a]	248±9[a]	248±5[a]	297±3[a]	297±3[a]	299±6[a]	261±3[a]	259±5[a]	263±6[a]
A-Cm (mg/l)	31±1[a]	32±2[a]	30±1[a]	46.0±0.7[a]	42.5±0.7[b]	42±1[b]	39±1[a]	37±1[a]	39.0±0.2[a]
A-Ac (mg/l)	13.3±0.8[a]	12.2±0.8[a]	13.5±0.2[a]	27±1[a]	15.7±0.7[b]	15.8±0.8[b]	29±1[a]	28.6±0.6[a]	29±1[a]
T-A (mg/l)	291±6[a]	292±9[a]	291±5[a]	370±3[a]	355±3[b]	357±6[b]	329±3[a]	324±5[a]	331±6[a]

Df, delfinidin; Ci, cyanidin; Pt, petunidin; Pn, peonidin; Mv, malvidin; Glc, glucoside; A-Glc, non-acylated anthocyanins; A-Ac, acetylated anthocyanins; A-Cm, *p*-cumarylated anthocyanins; A-T, total anthocyanins

All data are expressed as the arithmetic mean ± standard deviation (n =9). Means in the same column followed by the same letter indicate the absence of significant differences ($p>0.05$).

Table 5. Content of tannins (Tan) in the wines during barrel aging. Wine control (Control), wine in the presence of acidified lysate lees (LC), and wine in the presence of acidified lysate lees in combination with pectinases and β-glucanases (LCE).

	t = 0 days			t = 90 days		
	Control	LC	LCE	Control	LC	LCE
Tan (mg/l)	1786±106^{a}	1853±68^{a}	1906±67^{a}	2753±66^{a}	2219±90^{b}	2424±105^{b}

All data are expressed as the arithmetic mean ± standard deviation (n =9). Means in the same column followed by the same letter indicate the absence of significant differences ($p>0.05$).

Table 6. Color parameters in the wines during barrel aging. Wine control (Control), wine in the presence of acidified lysate lees (LC), and wine in the presence of acidified lysate lees in combination with pectinases and β-glucanases (LCE).

	t = 0 days			t = 45 days			t = 90 days		
	Control	LC	LCE	Control	LC	LCE	Control	LC	LCE
CI (UA)	13.3±0.3[a]	13.1±0.6[a]	13.0±0.5[a]	14.1±0.2[a]	14.2±0.5[a]	14.0±0.3[a]	14.4±0.2[a]	14.6±0.08[a]	15.1±0.3[b]
Hue	0.705±0.007[a]	0.709±0.005[a]	0.70±0.01[a]	0.68±0.01[a]	0.662±0.003[a]	0.665±0.008[a]	0.736±0.006[a]	0.734±0.004[a]	0.73±0.01[a]
A420 nm (UA)	4.8±0.1[a]	4.8±0.2[a]	4.7±0.2[a]	5.1±0.1[a]	5.0±0.2[a]	4.95±0.09[a]	5.37±0.03[a]	5.4±0.05[a]	5.6±0.2[a]
A520 nm (UA)	6.9±0.2[a]	6.7±0.3[a]	6.7±0.2[a]	7.41±0.05[a]	7.5±0.3[a]	7.4±0.1[a]	7.3±0.1[a]	7.41±0.03[a]	7.7±0.5[a]
A620 nm (UA)	1.57±0.04[a]	1.55±0.09[a]	1.53±0.09[a]	1.68±0.04[a]	1.66±0.06[a]	1.64±0.05[a]	1.7±0.02[a]	1.74±0.02[a]	1.80±0.09[a]
A280 nm (UA)	66±1[a]	65.0±0.8[b]	65±1.5[ab]	70±1[a]	68.8±0.9[b]	68.3±0.6[b]	67.0±0.7[a]	67±1[a]	66±2[a]
*L**	9.4±0.5[a]	10.0±0.6[a]	10.4±0.6[a]	7.8±0.5[a]	9.13±0.03[b]	9.1±0.2[b]	7.9±0.4[a]	7.2±0.2[a]	6.0±0.3[b]
C*	43±1[a]	44±1[a]	45±1[a]	40±1[a]	42.815±0.007[b]	42.6±0.5[b]	40.3±0.9[a]	38.8±0.5[a]	36.9±0.5[b]
H*	22.3±0.9[a]	23±1[a]	24±1[a]	19.6±0.7[a]	21.69±0.08[b]	21.6±0.2[b]	19.8±0.6[a]	19.0±0.3[a]	17.0±0.5[b]

All data are expressed as the arithmetic mean ± standard deviation (n =9). Means in the same column followed by the same letter indicate the absence of significant differences ($p>0.05$). CI, color intensity as sum of absorbances at 420, 520 and 620. Hue, A_{420}/A_{520}. *L**, luminosity; *C**,chromaticy; *H**, hue.

Figure 1.

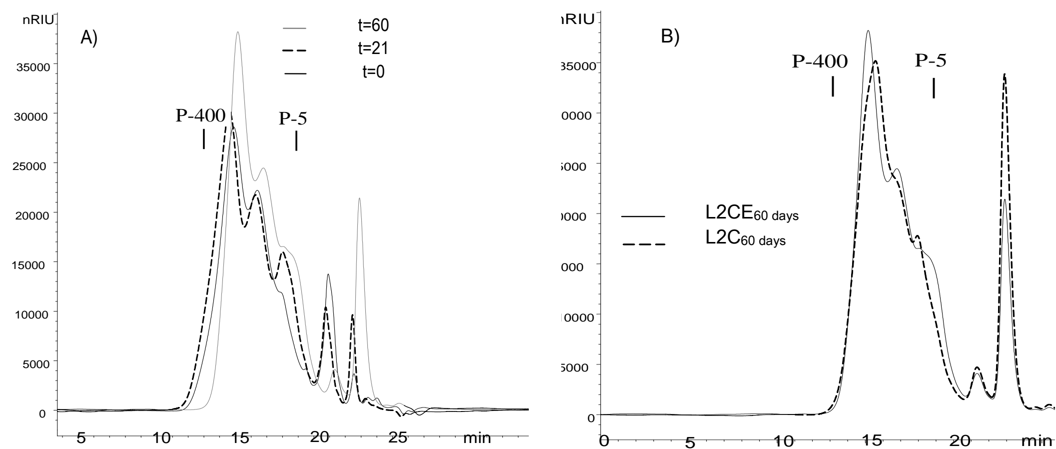

Figure 2.