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Adsorption of Phenolic Compounds and Browning Products in White Wines by Yeasts and Their Cell Walls

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Dehydrated yeast cells at variable concentrations were used as fining agents to decrease the color of white wines with two different degrees of browning (0.153 and 0.177 au, measured at 420 nm). Both wines showed a linear decrease of browning with increasing yeast concentration. However, in terms of efficiency, the yeasts exhibited a higher color lightening at greater concentrations acting on the darker wine. This suggests a preferential retention of some types of yellow-brown compounds that could increase their concentrations at the higher degree of browning. To confirm the role of yeast cell walls in the retention of browning compounds and to evaluate their potential use as fining agents, they were applied at variable concentrations to a browned wine (0.175 au). The cell walls were found to be the active support for the adsorption of browning compounds, but their efficiency was much lower than that of an equivalent amount of the yeast cells from which they were obtained. Finally, HPLC determinations of low-molecular-weight phenolic compounds showed flavan-3-ol derivatives to be significantly retained by both yeasts and their cell walls.

KEYWORDS: Browning; yeast; cell wall; white wine

INTRODUCTION

Color darkening during storage of white wines results essentially from chemical reactions involving phenolic compounds, particularly flavan-3-ol derivatives (*1*−*5*). Browning originates mainly from the oxidation of phenols, a slow process at the typical pH of wine, although it is accelerated by the presence of metals such as iron or copper (*5*−*8*). On the other hand, oxidative reactions of other wine compounds, such as the conversion of tartaric acid to glyoxilic acid, contribute to increasing the yellow color of wine, because glyoxal induces the condensation of flavans to colorless compounds, subsequently yielding yellowish substances (*9*−*11*). Other compounds such as acetaldehyde give similar condensation reactions (*12*).

Although the contribution of each of the previous reactions to the browning is unknown, their combined effect darkens the wine and shortens its commercial life to a variable extent. To reduce the concentration of brown compounds, the winemaking industry uses several fining agents, particularly active charcoal and polyvinylpolypyrrolidone (PVPP). These products are efficient for their intended purpose but can alter the sensory properties of wine if they are used in high concentrations (*13*−*15*). For this reason, beyond a given browning level, lightening the color of wine while maintaining its quality is very difficult.

Yeast cells are known to adsorb several compounds in the wine. Regarding phenolic compounds, some authors point out the ability of the yeasts to retain anthocyanins (*16*−*18*). This allows them to decolorize stained must ("moûts tachés") from red grapes (*19*). Similarly, yeast lees have been found to strongly interact with free polyphenols in model solutions (*20*). Also, some yeasts have been found to adsorb the compounds that are the precursors of the pinking in slightly oxidized white wines (*21*). On the basis of the foregoing, Bonilla et al. (*22*) recently demonstrated the potential of yeast cells as fining agents for color correction in sherry white wine as an alternative to active charcoal and PVPP, without alteration of sensorial properties.

The purpose of this work is to obtain a deeper knowledge of the adsorption of phenolic compounds and their browning derivatives by yeasts, as well as the alternative use of yeast cell walls as fining agents for the correction of browning in white wines.

MATERIAL AND METHODS

Samples. Two unclarified sherry type white wines, subjected to biological aging for 4 years, were supplied by an industrial winery of the Montilla-Moriles region (southern Spain) with an ethanol content of 15.5% (v/v) and were subjected to different fining treatments for correcting browning. The wines showed initial absorbances at 420 nm of 0.153 and 0.175 au. The first wine was divided in two batches, the first batch for use directly in the treatments and the second for use after natural browning by storage in contact with air at 20 °C until its absorbance reached 0.177 au.

Experiments. In a first experiment, 1-L samples of the first wine with the two different degrees of browning (0.153 and 0.177 au at 420 nm) were treated in triplicate in 45-cm-high × 6-cm-wide cylindrical containers directly with baker's yeasts (Mauripan Fleischmann's,

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Canada) in the dehydrated form at concentrations of 0.8, 1.2, 1.6, 2.0, 2.4, and 2.8 g/L. For better homogenization, after addition of yeasts the samples were stirred and then left to stand for 24 h at 20 °C. After this time of contact with the yeasts, the samples were passed through a MF-Millipore filter (nitrate and acetate cellulose) of 0.45-μm pore size.

To obtain cell walls for use in a second experiment, a suspension of the same strain of dehydrated yeasts mentioned above was broken by stirring for 5 min at 4 °C with an identical volume of glass beads of 0.5-mm diameter. The extracts thus obtained were centrifuged at 1200*g* and 4 °C for 15 min (Heraeus Sepatech Biofuge 17RS). The solid sediment, containing cell walls and whole cells, was suspended in water and subjected to a 500*g* pulse for 20 s to separate the whole cells. These were successively subjected to the above-mentioned treatment until they were totally broken. The fluid supernatant, containing the cell walls, was centrifuged at 1200*g* and 4 °C for 15 min and washed twice with distilled water. Because the sediment exhibited a high moisture, and its drying could alter the potential phenols' adsorption capacity, the equivalent of dry weight per milliliter of sediment was determined in an aliquot by drying at 104 °C to a constant weight. The dry weight/volume ratio thus obtained was used to calculate the cell wall concentrations to be employed in terms of sediment volumes (0.0677 g dry weight/mL of suspension).

For the development of the second experiment, samples of the second wine (A_{420} = 0.175 au) were treated in triplicate under the above-described conditions with yeast cell walls. Because this second experiment was designed after the acquisition of the results from the first experiment, the above-mentioned second wine was necessary. This wine was of the same type and a similar browning degree as the previous one. It should be pointed out that the objective of this work was to compare the efficiency of the dehydrated yeast with that of yeast cell walls. Taking into account that the above-mentioned procedure for the separation of cell walls can lead to variable loss (because of the need for several washes, centrifugations, etc.) and that the conditions of hydration could be different, it is imperative that the weight of the cell walls used for the treatments was the same when they were part of the whole cells as when they were free. Therefore, the cell walls corresponding to the doses of dehydrated yeasts for use in the treatment of the first wine were also separated, and their weights were used as doses in this second wine. For example, the weight of the cell walls separated from 2.8 g of dehydrated yeasts was 0.338 g. This weight corresponds to 1.98 g of dehydrated yeasts, because in the two separations of cell walls the losses were different. In this way, the cell wall concentrations used for the treatments of the second wine were 0.102, 0.149, 0.196, 0.244, 0.291, and 0.338 g dry weight/L (corresponding to 0.597, 0.876, 1.15, 1.43, 1.71, and 1.98 g/L of dehydrated yeasts, respectively). After 24 h of contact at 20 °C, the samples were passed through a MF-Millipore filter (nitrate and acetate cellulose) of 0.22-μm pore size.

Analytical Procedures. Spectrophotometric measurements at 420 nm were made on a Beckman spectrophotometer, model DU 600, with a 10-mm path length.

Extraction of Phenolic Compounds. A volume of 100 mL of wine was concentrated in a vacuum at 40 °C to 20 mL and was then adjusted to pH 7 with 0.1 M NaOH. The concentrate was passed through a Sep-Pak C_{18} cartridge with 900 mg of filling (Long Body Sep-Pak Plus, Waters Associates) that was previously activated with 8 mL of methanol and washed with distilled water, which was adjusted to pH 7 with NaOH according to Jaworski and Lee (*23*). The cartridge was eluted with 8 mL of water at pH 7. This volume, in addition to the volume obtained as a result of the sample run-through prior to the elution, was used for the determination of phenolic acid fraction. After preconditioning of the cartridge with 2 mL of water at pH 2, the flavan-3-ol fraction was eluted with 8 mL of 16% acetonitrile in water at pH 2 (*24*).

The two collected fractions were concentrated and passed through a filter of 0.45-μm pore size for injection into a Spectra-Physics P4000 HPLC instrument.

Identification and HPLC Analysis. The identification of the phenolic compounds was achieved by comparing their retention times with the retention times of the standards and their UV spectra obtained by HPLC diode array spectroscopy (Spectra-Physics UV6000LP), and by calculation of UV absorbance ratios after co-injection of samples and standards (*25*). Commercial standards were purchased from Sigma-Aldrich Chemical Co. (Madrid, Spain) and Sarsynthese Co. (Genay, France). Caftaric and coutaric acids were isolated by the method described by Singleton et al. (*26*). Procyanidins were obtained from a grape seed extract according to Bourzeix et al. (*27*). The standards' purity was 95−99%. Each compound was quantified by comparison with a calibration curve obtained with the corresponding standard, except for the procyanidins B3 and B4, which were quantified as procyanidins B1.

Analyses were carried out on a LiChrospher 100 RP-18 column (250 mm × 4.6 mm, 5-μm particle size) using 2% aqueous acetic acid and acetonitrile as mobile phases at a flow rate of 2 mL/min and with detection at 280 nm.

Phenolic Acids Fraction. The elution phases for this fraction were as follow: gradient elution from 0 to 5% CH_3CN in 5 min, isocratic elution for 10 min, gradient elution up to 15% CH_3CN in 5 min, isocratic elution for 10 min, gradient elution up to 100% CH_3CN in 10 min, and isocratic elution for 10 min. In this fraction were quantified the following compounds: gallic acid, protocatechuic acid, vanillic acid, syringic acid, *trans*-*p*-coumaric acid, *trans*-caftaric acid, *cis*-coutaric acid, and tyrosol.

Flavan-3-ol Fraction. The elution phases for this fraction were as follow: gradient elution from 0 to 15% CH_3CN in 5 min, isocratic elution for 10 min, gradient elution up to 20% CH_3CN in 5 min, gradient elution up to 30% CH_3CN in 10 min, gradient elution up to 100% CH_3CN in 10 min, and isocratic elution for 10 min. In this fraction were quantified catechin, epicatechin, and procyanidins B1, B2, B3, and B4.

HPLC Direct Injection. To avoid possible retention by the Sep-Pak cartridge affecting the browning products, all the wine samples were subjected to direct injection after filtration through a 0.45-μm pore size filter. The chromatograms registered at 420 nm showed a group of overlapped peaks eluted at high retention times and absorbing at this wavelength, corresponding to colored compounds (named "grouped peaks"). The elution conditions were the same as those used for the phenolic acids fraction. Because these peaks showed a higher absorbance at 280 nm than at 420 nm, the former wavelength was used for their quantification (expressed as gallic acid).

Statistical Procedures. Simple regression and variance analyses were performed on the replicated samples by using the Statgraphics Statistical Computer Package (Statistical Graphics Corp.).

Table 1. Means and Standard Deviations of Absorbances at 420 nm for the Wines Subjected to Fining Treatments with Several Concentrations of Dehydrated Yeasts and Cell Walls

wines before treatment	treated wines		
A_{420} (au)	fining agent	concn (g/L)	A_{420} (au)
0.153 ± 0.002	yeasts	0.8	0.126 ± 0.002
		1.2	0.121 ± 0.001
		1.6	0.116 ± 0.002
		2.0	0.117 ± 0.001
		2.4	0.114 ± 0.001
		2.8	0.111 ± 0.000
0.177 ± 0.001	yeasts	0.8	0.149 ± 0.001
		1.2	0.144 ± 0.002
		1.6	0.141 ± 0.000
		2.0	0.137 ± 0.002
		2.4	0.133 ± 0.000
		2.8	0.131 ± 0.001
0.175 ± 0.001	cell walls	0.102	0.167 ± 0.000
		0.149	0.163 ± 0.001
		0.196	0.160 ± 0.000
		0.244	0.156 ± 0.000
		0.290	0.153 ± 0.000
		0.338	0.153 ± 0.000

RESULTS AND DISCUSSION

Table 1 shows the absorbance at 420 nm of the wines at the two browning degrees considered, as well as the results obtained

Table 2. Means and Standard Deviations of Phenolic Compounds Contents (mg/L) in Wines before and after Treatments with a Concentration of 2.8 g/L Dehydrated Yeasts[a]

	lower browning wine $A_{420} = 0.153 \pm 0.002$			higher browning wine $A_{420} = 0.177 \pm 0.001$		
	before treatment	after treatment		before treatment	after treatment	
gallic acid	4.42 ± 0.31	4.29 ± 0.43		4.17 ± 0.40	4.13 ± 0.24	
protocatechuic acid	5.16 ± 0.88	4.23 ± 0.22		4.66 ± 0.27	4.42 ± 0.33	
vanillic acid	1.65 ± 0.11	1.59 ± 0.13		1.50 ± 0.05	1.36 ± 0.07	
syringic acid	1.40 ± 0.12	1.30 ± 0.06		1.19 ± 0.04	1.15 ± 0.05	
p-coumaric acid	0.581 ± 0.067	0.551 ± 0.027		0.617 ± 0.059	0.592 ± 0.031	
trans-caftaric acid	11.8 ± 1.0	11.3 ± 0.5		12.8 ± 0.2	11.8 ± 0.4	*
cis-coutaric acid	7.87 ± 0.71	7.42 ± 0.29		6.93 ± 0.73	6.45 ± 0.24	
tyrosol	54.4 ± 1.4	53.4 ± 7.8		56.5 ± 4.4	55.8 ± 0.9	
catechin	29.6 ± 1.0	26.5 ± 1.2	*	27.2 ± 1.0	23.8 ± 1.0	*
epicatechin	17.4 ± 0.7	15.3 ± 0.5	*	11.9 ± 0.1	9.94 ± 0.44	*
procyanidin B1	14.7 ± 1.3	12.2 ± 0.7	*	15.0 ± 0.4	13.4 ± 0.6	*
procyanidin B2	3.88 ± 0.74	3.12 ± 0.56		4.09 ± 0.25	3.32 ± 0.58	
procyanidin B3	17.2 ± 0.3	15.6 ± 0.3	*	16.2 ± 0.4	10.9 ± 1.5	*
procyanidin B4	4.55 ± 0.31	4.19 ± 0.39		5.72 ± 0.92	4.65 ± 0.74	
grouped peaks	13.6 ± 0.8	10.2 ± 0.6	*	16.3 ± 0.3	10.3 ± 0.5	*

[a] Values marked with an asterisk indicate significant differences obtained for each treatment ($p < 0.05$)

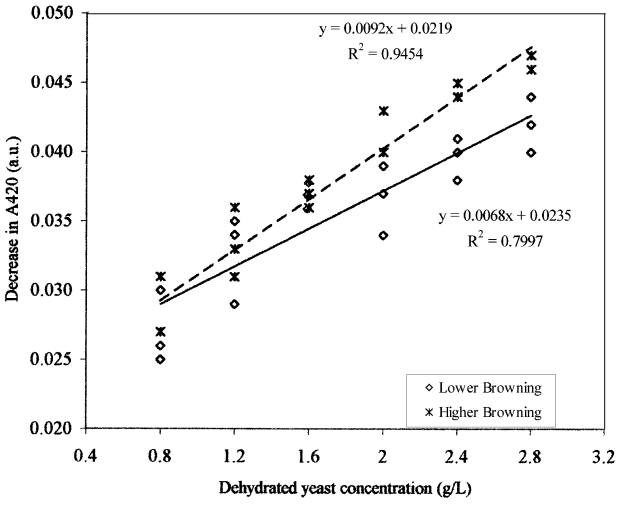

Figure 1. Decrease in A_{420} for the wine with the lower ($A_{420} = 0.153$ au) and higher ($A_{420} = 0.177$ au) browning degrees subjected to fining treatments with several concentrations of dehydrated yeasts.

after application of the different yeast concentrations used. As can be seen, all the doses of the yeast lightened the color of both wines. For the lower browned wine, several concentrations of yeast led to an absorbance level within or close to the standard value at the time of bottling of sherry wines (fixed in industrial winemaking at 0.110−0.115 au). However, for the higher browned wine, even the higher yeast concentration (2.8 g/L) was not sufficient to reach the above-mentioned standard value, although the decrease in absorbance obtained with this dose was similar in both wines. Therefore, because the initial browning of wines can be different, it is difficult to use the absolute values of absorbance to measure the efficiency of the yeasts, which is preferable to calculating their decreases.

Figure 1 shows the decreases in A_{420} as a function of the yeast concentration used. The results obtained for each wine were fitted by linear regression and found to exhibit a significant correlation ($p < 0.001$) in both cases. As can be seen, the absorbance decreases produced by yeast concentrations of 0.8 and 1.2 g/L were similar in both wines, which suggests a saturation in the retention capacity of the yeasts. On the other hand, as the yeast concentration was increased, the decreases in the absorbance were proportionally greater for the darker wine, proving that the yeasts did not saturate in the lighter wine. Therefore, the higher retention of brown compounds in the darker wine for the same concentration of yeasts suggests a specificity of these yeasts toward some types of colored compounds, which must increase their concentrations as browning develops. In this respect, some authors have shown flavans in model solutions to increase the brownish yellow color through their oxidation followed by polymerization and products resulting from their condensation with glyoxylic acid formed by oxidation of tartaric acid (*9*−*11*). The possible above-mentioned specificity of the yeasts could be reasonably related to a preferential retention of different compounds formed by diverse pathways or to compounds in different degrees of polymerization resulting from direct oxidation of phenols.

Table 2 lists the concentrations of phenolic compounds in the two wines, before and after treatments with the highest yeast concentration (2.8 g/L). It also shows a group of colored compounds (absorbing at 420 nm) obtained by direct injection in HPLC and eluted at high retention times, which were added and quantified as gallic acid at 280 nm, because they exhibited a higher absorbance at this wavelength than at 420 nm. Chromatograms corresponding to these colored compounds obtained on the darker wine before and after treatment at both wavelengths are shown in **Figure 2**. These so-called "grouped peaks" have been ascribed to phenols oxidation and/or condensation products, particularly flavans derivatives (*25*, *28*). In addition, **Table 2** gives the results of the analysis of variance performed for each compound and degree of browning, indicating those that exhibited significant differences resulting from the yeast treatment. As can be seen, the addition of yeasts reduced significantly the concentrations of catechin, epicatechin, procyanidins B1 and B3, and grouped peaks in both wines. Also, the concentration of *trans*-caftaric acid decreased significantly, although only in the darker wine. With the exception of this last non-flavonoid compound, these results show a particular ability of the yeasts to retain flavonoid phenols. These compounds have been identified as important contributors to wine browning, and some authors working on model solutions have found a correlation between peaks eluted at high retention times and the corresponding flavan monomer precursor (*5*, *28*). Therefore, it is reasonable to think that our grouped peaks may largely correspond to structures derived from the flavan-3-ol in a variable degree of polymerization.

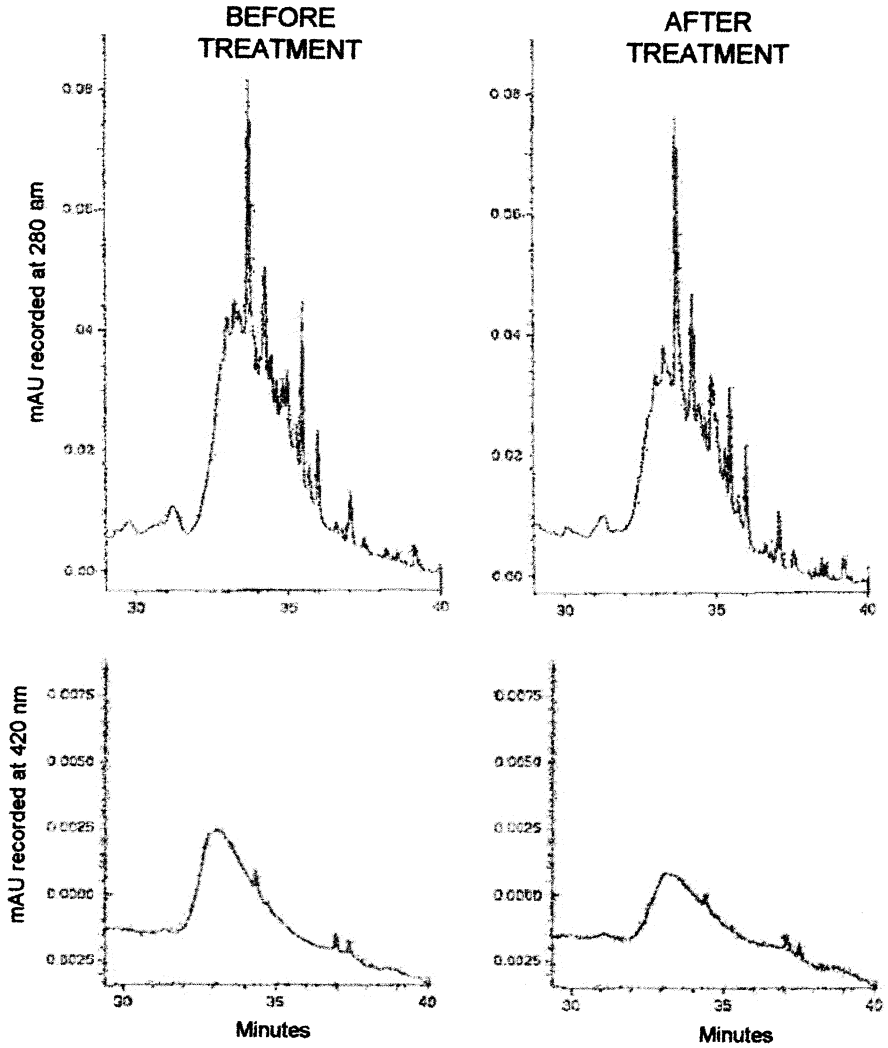

Figure 2. Chromatograms recorded at 280 and 420 nm of the "grouped peaks" obtained on the higher browned wine (A_{420} = 0.177 au), before and after treatment with 2.8 g/L of dehydrated yeasts.

Despite the proven efficiency of yeasts in correcting wine browning, the concentrations required to obtain acceptable results surpass those of other fining agents. For example, the clarification to a similar extent of the brown color of sherry white wine can require a yeast concentration 16 times higher than the amount of active charcoal required (*22*). Keeping in mind that cell walls account for 10−15% of the total yeast weight, and hypothesizing them to be the active support for the retention of phenols, the weight of clarifying agent used could be reduced by using cell walls instead of whole yeasts.

Figure 3 shows the A_{420} decrease observed in wines with a similar degree of browning treated with dehydrated yeasts and cell walls, respectively (**Table 1**). It must be pointed out that the dry weight of cell walls used (0.102, 0.149, 0.196, 0.244, 0.291, and 0.338 g) corresponded to the weight of dehydrated yeasts utilized as doses in the above-discussed experiment (0.8, 1.2, 1.6, 2.0, 2.4, and 2.8 g), in turn corresponding to 0.597, 0.876, 1.15, 1.43, 1.71, and 1.98 g of dehydrated yeasts because of the different losses resulting from the cell wall separation process carried out for this experiment. As can be seen, the regression equations obtained exhibit similar slopes, revealing the same behavior and, consequently, the ability of the cell walls to retain by adsorption colored compounds. However, their retaining efficiency is lower than that of the whole yeasts. Thus, a 2 g/L concentration of dehydrated yeasts decreased A_{420} by 0.04 au, while 0.338 g of cell walls (obtained from 1.98 g of yeasts) reduced it by only 0.02 au.

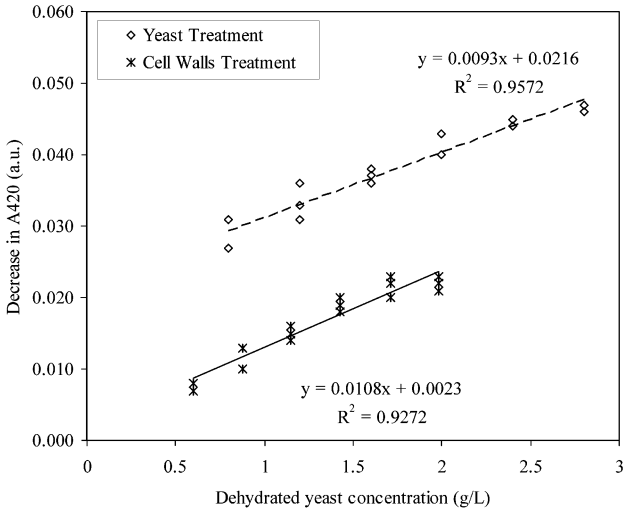

Figure 3. Decrease in A_{420} for the wines subjected to fining treatments with dehydrated yeasts and cell walls.

Although the experiments with whole dehydrated yeasts and cell walls were carried out with two different wines, the similarity between them, mainly in relation to the brown

Table 3. Means and Standard Deviations of Phenolic Compounds Contents (mg/L) before and after Treatment with a Concentration of 0.338 g/L Cell Walls[a]

	before treatment	after treatment	
gallic acid	3.91 ± 0.51	3.84 ± 0.57	
protocatechuic acid	5.09 ± 0.23	4.98 ± 0.13	
vanillic acid	1.26 ± 0.23	0.932 ± 0.008	
syringic acid	1.40 ± 0.10	1.35 ± 0.00	
p-coumaric acid	1.15 ± 0.09	1.05 ± 0.15	
trans-caftaric acid	11.7 ± 0.3	11.2 ± 0.3	
cis-coutaric acid	11.6 ± 0.2	11.3 ± 0.2	
tyrosol	79.9 ± 1.2	79.3 ± 1.9	
catechin	29.1 ± 1.3	26.0 ± 0.8	*
epicatechin	12.4 ± 1.2	8.42 ± 1.21	*
procyanidin B1	14.0 ± 0.6	11.4 ± 0.4	*
procyanidin B2	8.53 ± 1.10	3.73 ± 0.53	*
procyanidin B3	12.6 ± 1.4	9.84 ± 0.08	*
procyanidin B4	6.77 ± 0.26	5.88 ± 0.49	
grouped peaks	16.2 ± 1.4	12.1 ± 0.4	*

[a] Values marked with an asterisk indicate significant differences at $p < 0.05$

compounds, suggests that the differential efficiency observed should be ascribed to others causes. On one hand, the procedure used to disrupt the yeasts may cause their cell walls to break into fragments of variable size, potentially leading to interaction among them and reducing the number of active sites for retaining phenols as a result. On the other, the disruption of yeasts releases a large number of intracellular compounds, some of which might be retained on cell walls, additionally decreasing their phenol adsorption capacity. Likewise, the alteration in the charge of the cell wall resulting from its breaking could contribute to a decrease in its retention capacity. Finally, the possibility of partial intracellular penetration of brown compounds into the whole yeasts should not be discarded, as it has already been observed for some phenolic compounds (*29*). In this way, the yeasts could increase their efficiency in comparison to the cell walls, because these compounds would be eliminated with them in the filtration following the treatment.

However, on the basis of the experiments described above, in terms of dry weight, cell walls showed a better behavior than whole yeasts because one-sixth of the yeast weight of cell walls sufficed to yield 50% of the absorbance decrease achieved with whole yeasts. This suggests that the loss of efficiency in the cell walls is proportionally smaller than the weight difference involved.

Table 3 shows the phenolic compounds contents of the wines before and after treatment with the highest concentration of cell walls (0.338 g dry weight/L). The results showed retention of phenols by cell walls similar to that of whole yeasts (**Table 2**). Thus, except for procyanidin B2 in both wines treated with whole yeasts and *trans*-caftaric acid in the darker one, cell walls efficiently retained all flavan-3-ol derivatives studied, except for procyanidin B4, as well as the grouped peaks. These results confirm that yeast cell walls possess the ability to retain flavanols and their derivatives.

In conclusion, cell walls are responsible for the phenol retention capacity of yeasts. This capacity shows specificity to flavan-3-ol derivatives and colored products formed from phenolic oxidation or condensation reactions, or both. The latter products appear to be preferentially retained by the yeasts, judging from their behavior toward wines with different degrees of browning. Certainly, using cell walls rather than whole yeasts seemingly has no advantage because of the efficiency lost in the separation process. In addition, removing this cell fraction from the wine after treatment is more complicated than eliminating whole yeasts, which only require filtration through a filter of 0.45-μm pore size, common in the cellars.

A possibility for removing the fining agents (cell walls or yeasts) is by immobilization in gels with a large pellets size (1−2 mm) to prevent the formation of suspensions in the wine, therefore making them easy to separate by filtration. This, in turn, would allow color correction treatments to be conducted in a continuous process by circulating the wine through a bed of pellets, thus improving the efficiency. In this respect, using cell walls may be preferable on account of the smaller amounts required.

Based on the foregoing, further research is needed with a view to obtaining a deeper knowledge about the colored compounds in wine that are retained by yeasts and the related mechanisms. Taking into account the results obtained in this work, experiments involving model solutions containing flavans in variable degrees of degradation appear to point in the right direction for future research.

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