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Compositional changes during the storage of red wines treated with pectolytic enzymes: low molecular-weight phenols and flavan-3-ol derivative levels

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Abstract

The present work studies the effects of the addition of several commercial pectolytic enzyme products on the phenolic composition of red wine. Low-molecular-weight phenols and flavan-3-ol derivatives were studied. The results revealed that the addition of enzymes produced an overall increase in the extraction of these compounds. However, in the case of flavan-3-ol derivatives, extraction was increased only when sufficient time was allowed for maceration. Study of the evolution of the contents of these compounds over time suggested that, in general, wines that initially contain higher amounts of phenolic compounds are able to maintain higher levels of these compounds during storage.

Keywords: Red wine; Pectolytic enzymes; Flavan-3-ol; Low-weight-phenols; Storage; Stability

1. Introduction

Catechin and proanthocyanidins are phenolic compounds that influence the colour, astringency, bitterness, oxidation level, and clarity of wines and that are also involved in the changes that occur during wine aging (Singleton, 1992). Furthermore, catechin and proanthocyanidins contribute to the healthful properties of red wine, which is a dietary source of these compounds. These compounds act as antioxidants (Rice-Evans, Miller, & Paganga, 1996; Teissedre, Frankel, Waterhouse, Pleg, & German, 1996), scavenge free radicals (Arpentine, Fernández, Bourzeix & Mitjavila, 1992; Ricardo da Silva, Darmon, Fernández, & Mitjavila, 1991) induce vascular relaxation (Mendes, Desgranges, Vercauteren, & Freslon, 1999) and also have anti-inflammatory (Gábor, 1986), anticarcinogenic and antimutagenic properties (Liu & Castonguay, 1991; Liviero, Puglisi, Morazzoni, & Borbardelli, 1994). Such properties exert positive effects against a large variety of degenerative processes, such as cancer, atherosclerosis, cardiovascular diseases and aging.

Low-molecular-weight phenols (benzoic and cinnamic acids and aldehydes) are present in very low amounts in wines but they play important roles in determining their sensory qualities (Peynaud, 1989). Some reports have claimed that through an additive effect, they may contribute—especially cinnamic acids—to bitterness and harshness (Singleton & Noble, 1976). Also, cinnamic acids are involved in copigmentation processes with anthocyanins, which affect the colour of red wine (Brouillard & Dangles, 1994).

Catechin and proanthocyanidins are mainly found in grape seeds (Romeyer, Macheix, & Sapis, 1986) and their presence has also been reported in the skin (Escribano-Bailón, Guerra, Rivas-Gonzalo, & Santos-Buelga, 1995), whereas low-molecular-weight phenols are located in the skin and pulp, where benzoic and cinnamic acids predominate (Fernández de Simón, Estrella, Hernández, & Santa-Martía, 1990). All these compounds are extracted into the wine during grape fermentation and maceration. Among the principal factors affecting the phenolic content of wines are the winemaking pro-

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cess itself and the reactions that take place during aging (Macheix, Sapis, & Fleuriet, 1991).

Many studies have focused on the levels of these compounds and the effects of the technological process used for red wine production (Dallas, Ricardo da Silva, & Laureano, 1995; Kovac, Alonso, Bourzeix, & Revilla, 1992). The addition of pectolytic enzymes is a common practice in wineries to increase the phenolic content, especially anthocyanins, although their influence on proanthocyanidin and low-molecular-weight phenol contents has received less attention.

During storage and aging of red wines, polyphenolic compounds are gradually modified. Reactions among anthocyanins, flavan-3-ol, proanthocyanidins and other compounds, such as glyoxylic acid, pyruvic acid and acetaldehyde, and also between flavonols themselves, have been observed. These reactions are responsible for the appearance of new pigments (Bakker & Timberlake, 1997; Dallas Ricardo da Silva, & Laureano, 1996; Fulcrand, Benabdeljalil, Rigaud, Cheynier, & Moutonet, 1998, Revilla, Pérez-Magariño, González-SanJosé, & Beltrán, 1999), yellow xanthylium type polymers (Jurd & Somers, 1970) and hence for the disappearance of oligomer proanthocyanidin from the solution.

These processes affect colour and colloidal stability (Saucier, Bourgeois, Christiane, Roux, & Glories 1997) and also the nutraceutical properties. Few studies of the evolution of flavan-3-ol derivatives and low-molecular-weight phenols, or the effects of technological treatment during the wine maturation phase have been reported (Dallas et al., 1995) and, to our knowledge, there are none referring to the addition of pectolytic enzymes.

The aim of the present work was therefore to study the effect of this enological practice on the final levels of catechin, proanthocyanidin and low-weight-molecular phenols in treated wines and the evolution of the wine during the storage phase.

2. Materials and methods

2.1. Experimental material

Grapes of the "Tinto Fino" variety (*Vitis vinifera*) were harvested at commercial maturity (23–25 ° Brix). Two vintages were harvested and processed for two consecutive years. The damaged grape clusters (broken or with visual microbial alterations) were separated in order to eliminate undesirable contamination and degradation compounds. Groups of intact clusters were processed in small steel tanks.

2.2. Vinifications

2.2.1. Control

Once de-stemmed and crushed, and following the addition of 0.04 g/l of SO_2, the grape extract was inoculated with 0.1 g/l of previously hydrated commercial yeast—*S. cerevisiae* and *S. bayanus* (Wormser Oenologie). Alcoholic fermentation was then carried out at 25 °C. The end of fermentation was determined by the total consumption of reducing sugars.

At the end of the alcoholic fermentation period, the wine was strained off, the grape pomace was pressed ($P < 2$ kg/cm^2), and the resulting wine was transferred to another tank and kept at 1 °C for 48 h to facilitate the settling process. Then, the wine was centrifuged (10,500 *g* for 10 min at 5 °C) and the clarified wine was bottled in green glass bottles and stored at 15 °C for 2 years.

2.2.2. Vinification with enzymatic treatment

In this case, the only difference with the control treatment was the addition of pectolytic enzymes 1 h (the time suggested by the producers) before the inoculation of the yeast in order to improve the enzymatic action. During this time, the grape pomace was kept at 20 °C.

Four different commercial pectolytic enzymes were used at the maximum and minimum doses suggested by the producers. The type of enzymes and codes used were as follows: (a) *Clarifying pectolytic enzymes*: Zimopec PX1 (Perdomini SPA) 0.01 g/l (Z.1) and 0.03 g/l (Z.3), and Rapidase CX (DSM) 0.02 g/l (R.2) and 0.05 g/l (R.5); (b) *Colour-extracting enzymes*: Pectinase WL Extraction (Wormser Oenologie) 0.005 g/l (P.05) and 0.01 g/l (P.1), and Rapidase Ex. Colour (DSM) 0.02 g/l (R.ex.2) and 0.05 g/l (R.ex.5).

Each treatment type (control and enzymatic vinification) was performed in duplicate. Wine samples were taken at the end of the vinification process and every 6 months over the 2 years of storage.

2.3. Fractionation

Wine samples were extracted by column fractionation following the method proposed by Di Stefano and Cravero (1990), using Amberlite XAD-2 (Sigma) adsorbent resin. Ten millilitres of wine were acidified with 10 ml of 1N H_2SO_4 and then introduced into the column. Acids, sugars and other water-soluble compounds were eliminated with distilled water. Then, two fractions were eluted with the following solvents: diethyl ether (fraction 1), which mainly recovers low-molecular weight phenols, and ethyl acetate (fraction 2), which mainly affords catechins and proanthocyanidins. The column was then washed with acidified methanol and water. Each fraction was evaporated to dryness under vacuum, avoiding temperatures higher than 35 °C, and the residue was re-dissolved in 4 ml of methanol. Fractionation was carried out in duplicate. Samples were kept at −18 °C until analysis.

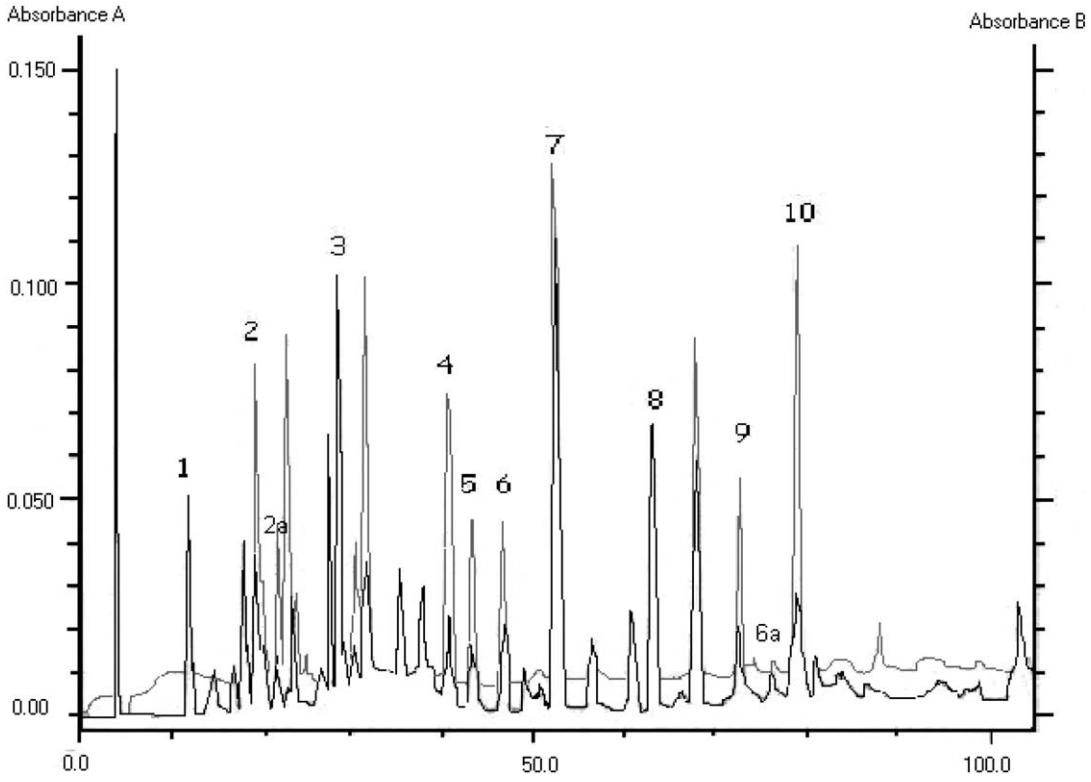


Fig. 1. HPLC chromatogram of low molecular-weight phenols (See Table 1 for peak identification).

2.4. High-performance liquid chromatography (HPLC)

Analyses were carried out using an HP 1100 Series, fitted with a 7125 Rheodyne injector (Fisons Instrument). The system was equipped with a Diode Array Detector. Samples were analysed on a reverse-phase column (Spherisorb ODS2, 250 mm × 4.6 mm ID, particle size 3 μm) at a constant temperature of 25 °C. Simultaneous detection was allowed at 254 and 280 nm and UV–vis spectra (scanning from 240 nm to 500 nm) were recorded for all peaks. Two methods were employed.

Low-molecular- weight phenols (F1). were quantified using the method proposed by Bartolomé, Bengoechea, Gálvez, Pèrez-Ilzarbe, Hernández, Estrella et al. (1993) modified to adapt it to the equipment used. Solvent A was water: acetic acid (98:2) and solvent B was water: methanol: acetic acid (68:30:2). Flow rate was 0.6 ml/min, with a linear gradient from 5 to 30% solvent B over 20 min; 30–55% over 25 min, 55–77% over 10 min and isocratic for 15 min. Following this, the gradient applied was linear from 77 to 95% over 5 min and from 95 to 100% over 10 min.

Samples were diluted with water (80:20) and filtered through a 0.45-μm pore-size membrane.

Retention times and spectral UV–vis features were used to identify the compounds, comparing them with injected standards, and each compound was quantified in the corresponding calibration curve. In addition, confirmation of the identity of the compounds was obtained by HPLC–MS detection (Fig. 1, Table 1).

Flavan-3-ol derivatives (F2) were analysed using the method proposed by Pérez-Magariño, Revilla, González-SanJosé, and Beltrán (1999). Solvent A was 4.5% formic acid in water and solvent B was solvent A:Acetonitrile (90:10), with a linear gradient from 0 to 50% solvent B over 25 min; 50–80% over 35 min and 80% isocratic for 20 min. Flow rate was 0.7 ml/min. The samples were diluted with water (20:80) and filtered through a 0.45-μm pore-size membrane. Catechin, epicatechin, epigalocatechin, epicatechin gallate and epigalocatechin gallate were used as standards (Sigma-Aldrich). The elution order and the spectral UV–vis and

Table 1
Low molecular-weight phenol detected in wine samples

Peak	Peak identity
1	Gallic acid
2	Protocatechuic acid
2 a	Protocatechuic aldehyde
3	Caftaric acid
4	Catechin
5	Coutaric acid
6	Vanillic acid
7	Caffeic acid
8	Ferulic acid
9	Epicatechin
10	*p*-Coumaric acid

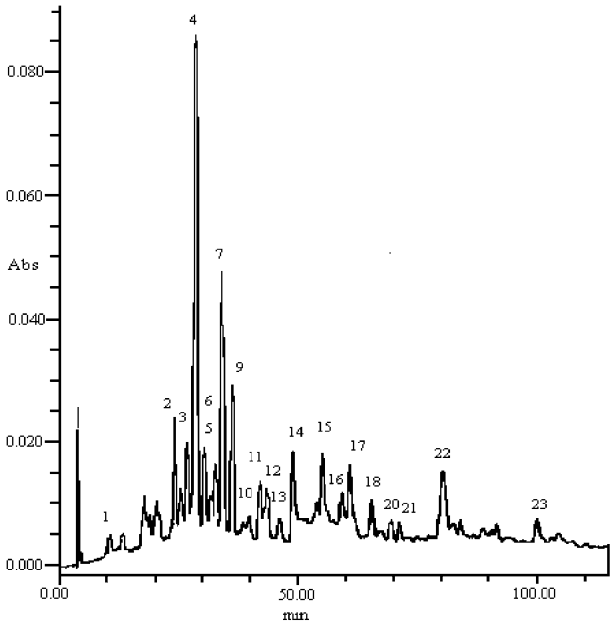


Fig. 2. HPLC chromatogram of flavan-3-ol derivatives (See Table 2 for peak identification).

Table 2
Flavan-3-ol derivatives detected in wine samples

Peak	Peak identity
1	Gallic acid
2	Caftaric acid
3	Trimer
4	Dimer
5	Dimer
6	Tetramer
7	Catechin
8	Epigalocatechin
9	Dimer
10	Trimer
11	Dimer
12	Dimer
13	Dimer
14	*p*-Coumaric acid
15	Galloyl derivative
16	Phenolic acid
17	Phenolic acid
18	Epicatechin
19	Epigalocatechin gallate
20	Galloyl derivative
21	Galloyl derivative
22	Tetramer
23	Epicatechin gallate

MS features of flavan-3-ol isolated derivatives were used for identification (Fig. 2, Table 2).

Milli-Q water was used throughout. HPLC-grade acetonitrile, methanol (Lab-Scan, Dublin, Ireland) and acetic and formic acid (Merck, Darmstadt, Germany) were used after filtration through a 0.45-μm pore-size membrane.

Varying amounts of sample were injected into the HPLC in order for them to be within the linear range of the standard curves.

2.5. Statistical analysis

Statistical treatment of the data was carried out by analysis of variance (ANOVA). The statistical significance of each factor considered was calculated at the $\alpha = 0.05$ level using the *F*-test. The LSD Fisher-test was employed to test for statistically significant differences between samples. All statistical analyses were carried out using the Statgraphic Plus for Windows Computer Package (1995 Manugistics, Inc.)

3. Results and discussion

3.1. Effects of enzymatic treatment on phenol composition

3.1.1. Low-molecular-weight-phenols

Figs. 3 and 4 show the mean contents of the different low-molecular-weight phenols quantified in recently made wine. As can be seen, the total contents of phenolic aldehydes were similar for both vintages, vanillic aldehyde being the most abundant. Statistical analysis of the data revealed that the addition of pectolytic enzymes elicited a significant increase in vanillic aldehyde in both vintages. The most effective preparations for its extraction were the extracting-type pectolytic enzymes (P and R.ex).

It should be noted that the contents in the phenolic acids varied as a function of the vintage, the caftaric acid being the most abundant for the first vintage and ferulic acid in the case of the second. Indeed, caftaric acid was present at higher levels in the first vintage than in the second one, while ferulic acid showed higher contents in the second vintage. These differences are due to the effect of climatic conditions on the composition of the grape.

It is not clear what effect enzymatic addition had, since it varied from compound to compound and in each vintage and only in the case of the most abundant acids present in each vintage was the effect of enzymatic addition clear. Wines treated with extracting enzymes and R.5 showed statistically significant higher levels than the controls.

3.1.2. Flavan-3-ol monomers and polymers

Flavan-3-ol derivatives were grouped, according to their degree of polymerization, into monomers, dimers, trimers, tetramers, and galloyl derivatives (Fig. 5). According to the data, the treated wines were richer in catechin than in epicatechin. On the other hand, of all the polymers studied the dimers were the most abun-

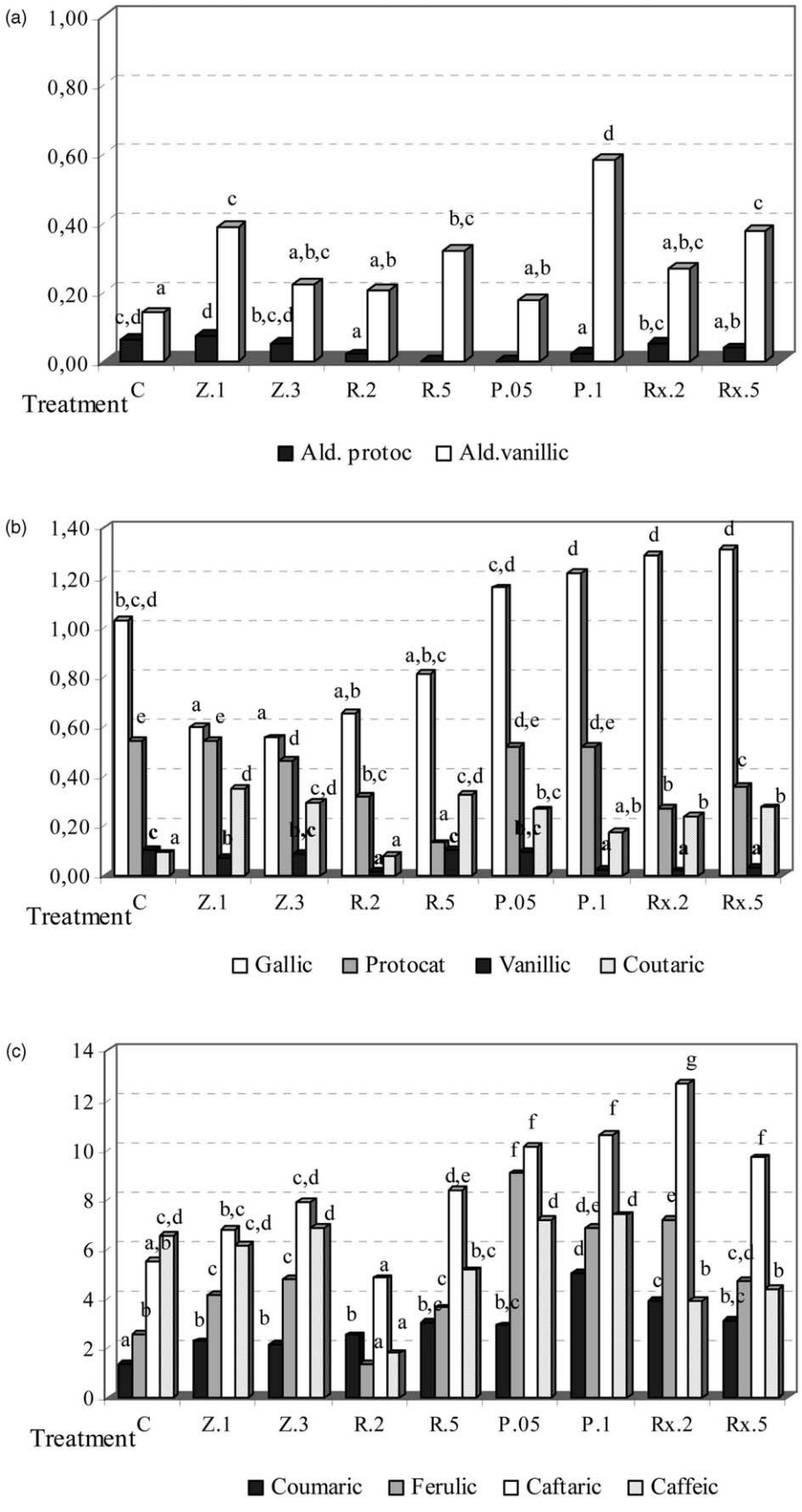
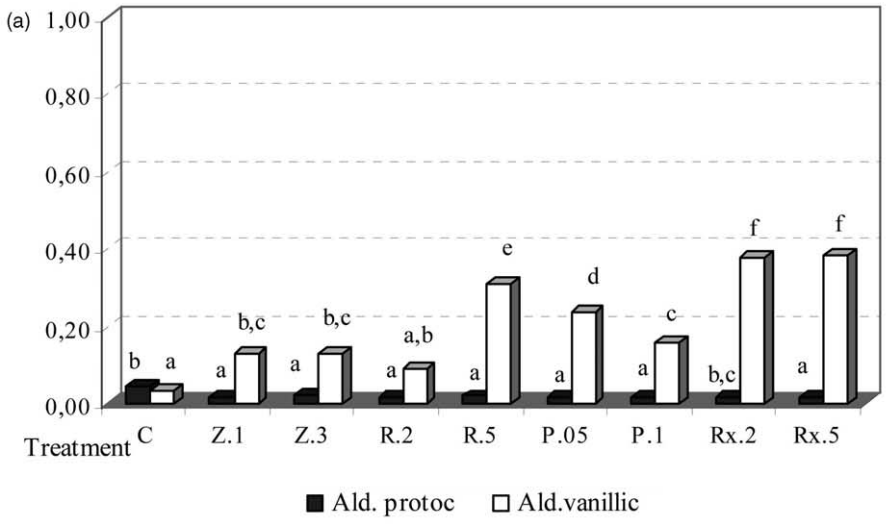
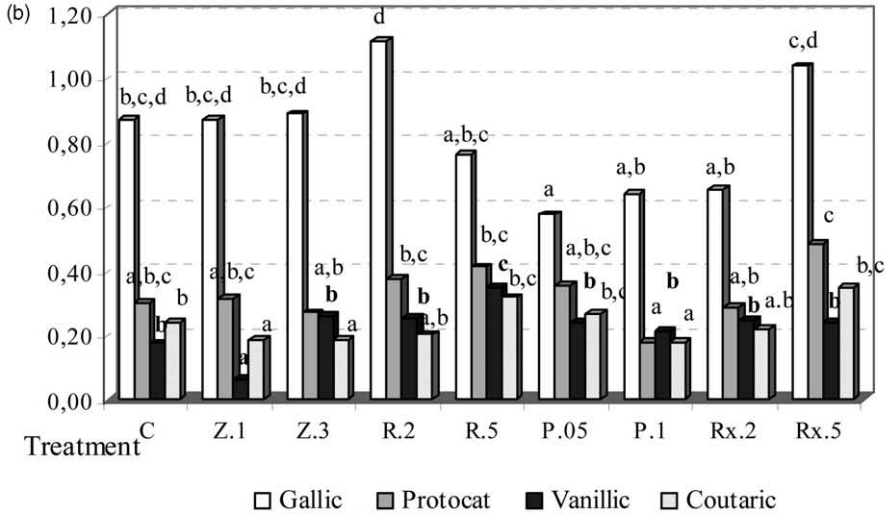


Fig. 3. Mean values of low-molecular weight phenols, expressed in $mg \cdot l^{-1}$, in recently elaborated first vintage wines: (a) phenolic aldehydes, (b) and (c) phenolic acids.





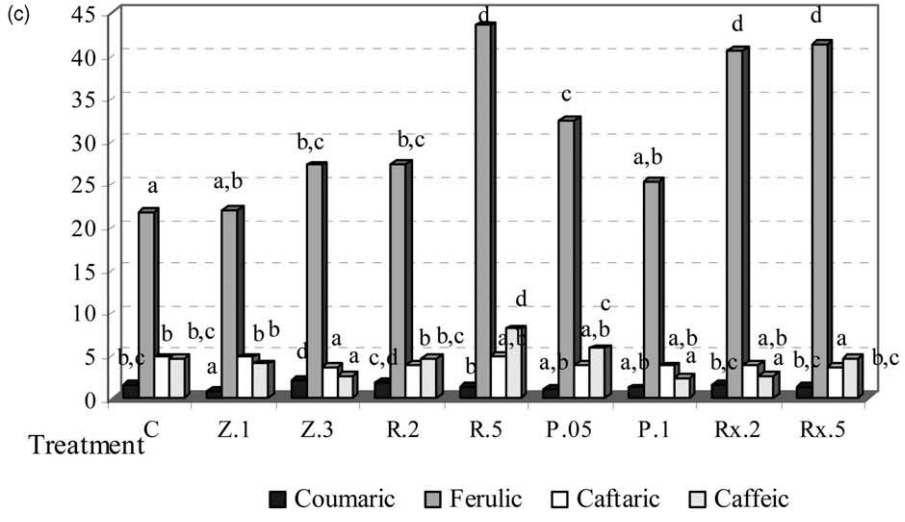


Fig. 4. Mean values of low-molecular weight phenols, expressed in $mg \cdot l^{-1}$, in recently elaborated second vintage wines: (a) phenolic aldehydes, (b) and (c) phenolic acids.

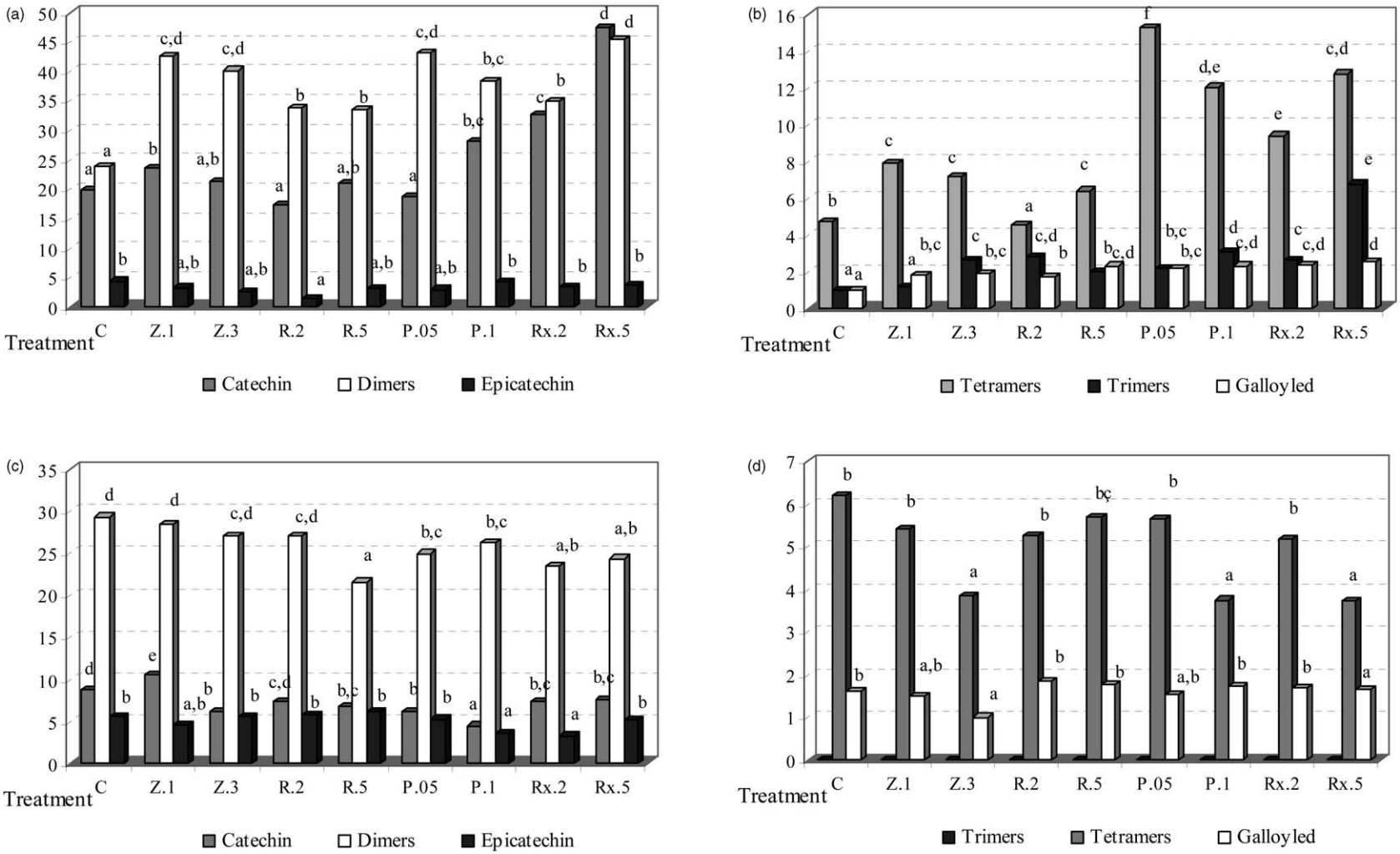


Fig. 5. Mean values of flavan-3-ol derivatives, expressed in $mg \cdot l^{-1}$, in recently elaborated wines: (a) and (b) first vintage; (c) and (d) second vintage.

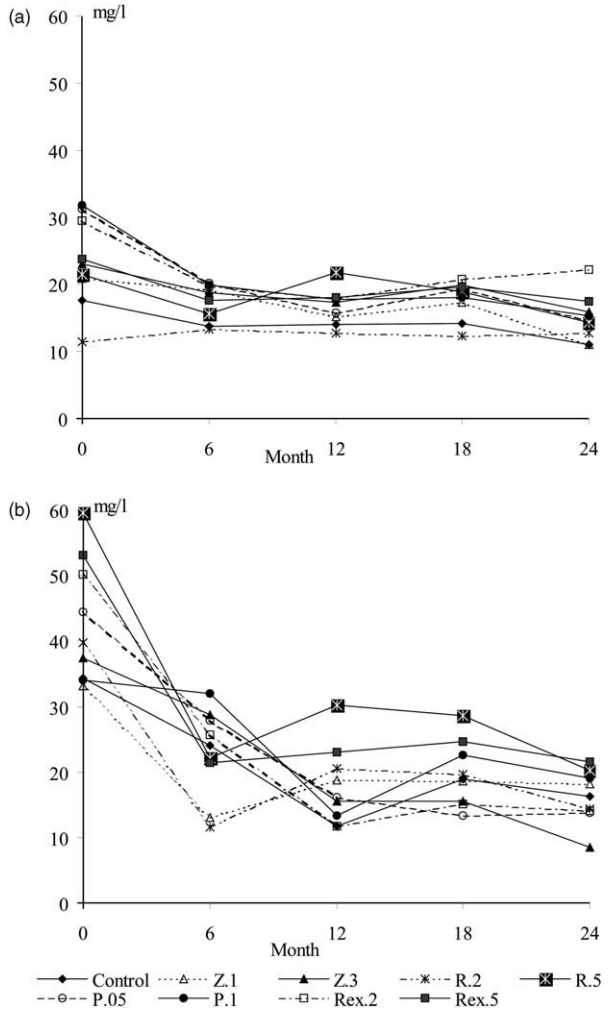


Fig. 6. Evolution of phenolic acids during storage in wines: (a) first vintage, (b) second vintage (for each treatment mean values of four replicates are shown).

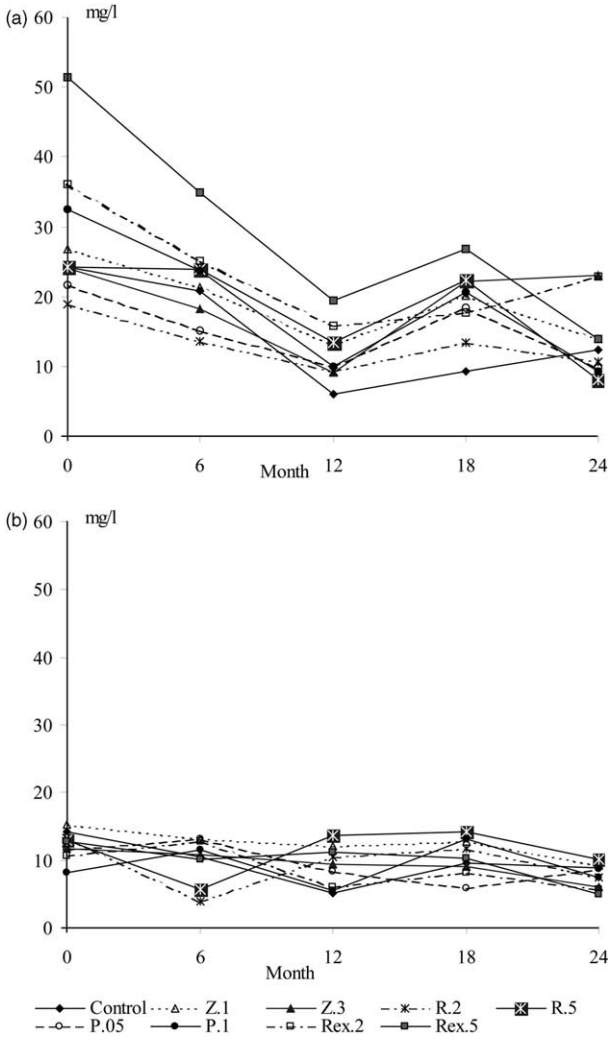


Fig. 7. Evolution of flavan-3-ol monomers during storage in wines: (a) first vintage, (b) second vintage (for each treatment, mean values of four replicates are shown).

dant and, in the recently made wines from the second vintage, no trimers were detected.

The data concerning the wines from the first year suggest a positive effect resulting from the addition of pectolytic enzymes, especially for extracting preparations. This is reflected in an increase in the levels of all groups analysed except for epicatechin.

These effects could not be corroborated in the following vintage since no beneficial effect was observed in the wines from the second year. It should be noted that the fermentation of this year occurred very rapidly, resulting in a very short maceration time; this could explain why the enzymatic effect was not detected. It should be taken into account that these compounds are located in the solid parts of the grape (skin and mainly in the seeds) and hence the duration of the maceration process has an important effect on their extraction.

3.2. Evolution of phenolic compound levels in wine during storage (ageing)

3.2.1. Low-molecular-weight phenols

The evolution of the contents of phenolic acids and the differences between treatments was followed as the sum of the different phenolic acids quantified. Fig. 6 (a and b) shows this evolution, which was similar for both vintages under study and was characterized by a decrease in the levels of phenolic acids during the first 6–12 months, later levelling off to more or less constant values. With this type of compound, evolution was more or less parallel for all the treatments applied, such that, the higher the initial contents, the greater the levels observed during storage. Because phenolic acids can act as anthocyanin copigments, stabilizing the wine colour, higher contents of these compounds will have a positive effect on the colour.

As in the case of the phenolic acids, the sum of the phenolic aldehydes was calculated. Their evolution was similar to the phenolic acids, but with greater losses in the initial phase of storage. Thus, in order to simplify the results, these data are not shown graphically.

3.2.2. Flavan-3-ol monomers and polymers

The different compounds were grouped according to the degree of polymerization. The results for the flavan-3-ol monomers (catechin and epicatechin) in the first year pointed to a gradual loss during the first twelve months (Fig. 7a and b). The wines with the highest initial concentrations exhibited the greatest losses. Despite this, the enzymatically treated wines showed higher concentrations of these compounds than the control wine.

In the second vintage, all wines starting with similar values followed a parallel evolution, characterized by a slight tendency to decrease. Comparing these with the first vintage wines, it is possible to conclude that the losses were smaller when the initial concentrations were lower.

Similar results were obtained in the evolution of the flavan-3-ol dimers, characterized by an initial drop and subsequent stabilization. The enzymatically treated wines were the richest in this kind of compound.

The drop in these two groups of phenols is due to the fact that there are highly reactive compounds which are involved in condensation and polymerization processes by themselves and with anthocyanins, as well as in oxidation reactions.

The evolution of the trimers was characterized by continuous changes in levels that could be correlated with dynamic processes of formation from the flavan-3-ol monomers and dimer polimerization products and the disappearing due to the continuous polymerization process. The lower content of flavan-3-ol compounds of second vintage wines could be why, initially, these compounds were not detected in this vintage (Fig. 8 a).

The evolution of the flavan-3-ol tetramers followed different types of behaviour, depending on the vintage. The first vintage was characterized by an increase at 6–12 months, depending on the treatment applied (Fig. 8b). This could be the result of the polymerization of the structures with a lower degree of condensation. Following this, the continuous increase in the degree of polymerization led to their precipitation, causing a decrease in these levels. In general, the fact that the enzymatically treated wines showed greater values in these compounds could be because they had higher levels of polymerization substrates. Lower concentrations were detected in the second vintage wines. In this case, all the wines exhibited parallel evolutions with a slight tendency to decrease.

Finally, the galloyl derivatives did not show important changes during storage, their levels remaining more or less constant. This could indicate that these compounds were very stable.

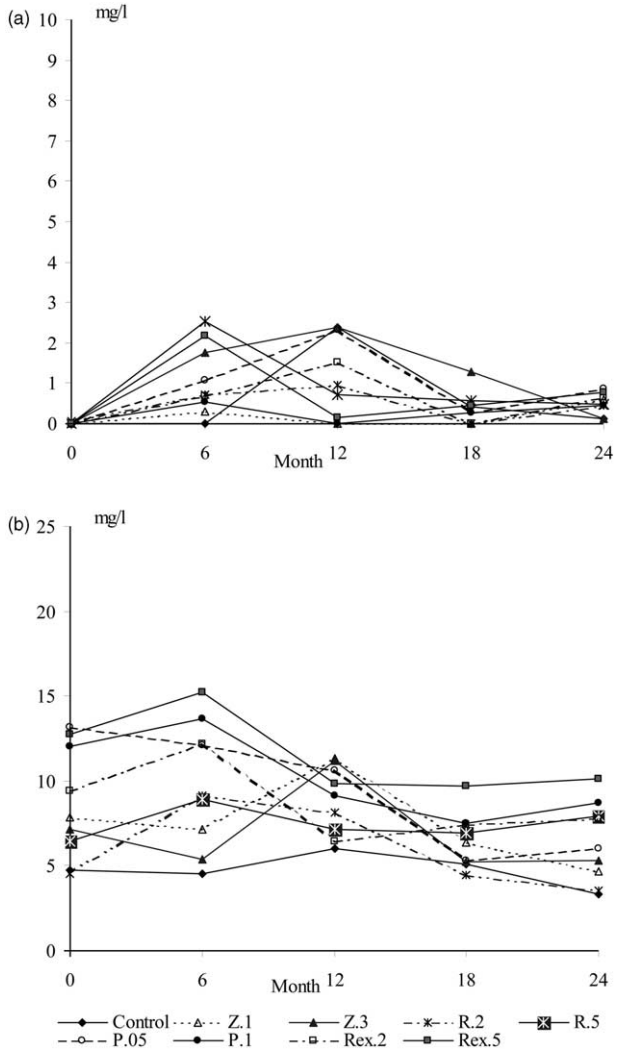


Fig. 8. Evolution of flavan-3-ol polymers (a) trimers (2 vintage) and (b) tetramers (1st vintage).

4. Conclusion

The addition of pectolytic preparations, particularly extractor types, increases the total contents of phenolic aldehydes and acids. On the other hand, a certain tendency of the levels of flavan-3-ol monomers and polymers to increase was observed, although this was strongly dependent on the duration of the maceration.

The addition of pectolytic enzymes constitutes an avenue of enrichment for red wine in this type of compound; furthermore, they are are maintained in higher quantities during the storage of these wines.

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