Influence of Prefermentation Clarification on Heavy Metal Lability in Garnacha Must and Rosé Wine Using Differential Pulse Anodic Stripping Voltammetry

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A desirable must clarification process should improve the quality of the wine, remove substances that produce unwanted flavors, favor the fermentation to dryness, and increase the fermentation rate. Among the factors that affect the quality of wine, the cationic content and the form in which the cations exist in the must are important since they play a relevant part in the fermentative process. Differential pulse anodic stripping voltammetry (DPASV) is a precise and inexpensive technique for metal analysis, has excellent sensitivity due to preconcentration of metal ions in the mercury drop or film, and offers the potential of discriminating between certain electroactive and electroinactive metal forms. The aim of this study was to observe the influence of two prefermentation clarification treatments, static sedimentation and vacuum filtration, on total, acid-displaceable, and labile heavy metal content. The results demonstrate that vacuum filtration of the must decreases Zn and Cu, as much for total metal as well as labile species and those displaced by acid. In filtered must, the percentage of labile Zn and Cu increased with respect to its total metal content. However, sedimentation did not eliminate metal species displaced at pH 2. Cadmium and lead, with elevated toxicity, were greatly eliminated by clarification; what remained in the must formed highly stable complexes that cannot be measured with DPASV.

Keywords: *DPASV; heavy metals; prefermentation clarification; Garnacha must; rosé wine*

INTRODUCTION

A desirable must clarification process should improve the quality of the wine, remove substances that produce unwanted flavors, favor the fermentation to dryness, and increase the fermentation rate (Groat and Ough, 1978). When clarification is excessive, especially in musts from grapes in good condition, it can affect nutrients and factors decisive in the fermentation and cause diverse problems in the wine, such as elevated concentrations of acetic acid, which destroys the quality of the product (Delfini and Cervetti, 1987, 1988; Delfini and Costa, 1993). Among the factors that affect wine quality, cationic content and the form in which the cations exist are fundamental since they are important in the fermentative process (Ribérau-Gayon et al., 1976). In must, some metals (Zn, Cu, Fe) at low concentrations are cofactors for vitamins and enzymes, but others, like Pb, Hg, Cd, and Se, are toxic (Eschnauer, 1982; Fernández Pereira, 1988).

Differential pulse anodic stripping voltammetry (DPASV), besides being a precise and inexpensive technique for the analysis of metals in must and wine, has excellent sensitivity due to preconcentration of metal ions in the mercury drop or film (Bond, 1980; Riley and Watson, 1987; Bersier, 1987; Willard et al., 1988). The fraction of a metal in solution that is DPASV-labile (i.e., reactive at the electrode) depends on the kinetics of metal complex dissociation during the deposition step, the excess concentration of ligand, and the diffusion-layer thickness (Florence and Mann, 1987). The deposited species, collectively called "labile" (Buffle,

1988), are those which are in labile equilibrium or dissociated within the time scale of the measurement. This fraction will therefore be dependent on pH, the selected deposition potential, the electrode system, and its condition of operation. The presence of complexing agents or surface-active substances in the sample may be important in the stripping process because of the large excess of the oxidized metal ion (compared with the bulk solution) that is present at the surface of the electrode during the initial stages of stripping. This excess can cause the precipitation of metal compounds on the electrode which may then affect the stripping current (Florence and Mann, 1987).

To obtain meaningful speciation results, careful consideration of operating conditions is required. Voltammetric measurements at natural pH values greater then 7 are insensitive to certain electroinactive ionic metal species such as lead and cadmium hydroxycarbonates. Acidification to pH 2 may dissociate some organic complexes or release metals adsorbed on colloidal species. However, measurements at such extreme pH values have been used in speciation studies (Batley, 1983; Pickering, 1995). By its nature, DPASV can provide an operationally-defined measurement of labile metal. Qualitative determination of elements is carried out through peak potential, E_p, whereas quantification is obtained from peak height, i_p, which, under ideal experimental conditions, is proportional to concentration.

The aim of this study was to observe the influence of two prefermentation clarification treatments, static sedimentation and vacuum filtration, on (a) total content of Zn, Cd, Pb, and Cu, (b) acid-displaceable metal, and (c) labile metal. An additional objective was to compare the influence of deposition time, t_d, and sample volume, V_s, on i_p and E_p. This study was done with Garnacha must clarified by two different prefermenta-

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Table 1. Characteristics of Garnacha Musts and Rosé Wines[a]

sample	turbidity (NTU ± SE)	pH ± SE	reducing sugar (g/L ± SE)	volatile acidity (g/L[b] ± SE)	ammonium nitrogen (mg/L ± SE)	total higher alcohols (mg/L ± SE)
			Musts			
control	1460 ± 14	3.31 ± 0.01	205 ± 1		107.4 ± 0.7	
decanted	205 ± 5	3.30 ± 0.01	202.3 ± 0.6		110.6 ± 0.6	
filtered	66 ± 1	3.31 ± 0.01	204 ± 3		116.0 ± 0.7	
			Wines from Musts			
control		3.12 ± 0.01	0.96 ± 0.01	0.27 ± 0.04	4.7 ± 0.1	241 ± 13
decanted		3.10 ± 0.01	0.99 ± 0.05	0.30 ± 0.02	4.7 ± 0.1	203 ± 8
filtered		3.33 ± 0.01	0.76 ± 0.02	0.74 ± 0.03	24.4 ± 0.1	99 ± 2

[a] $n = 4$ and all parameters with their standard error, SE. [b] In g of acetic acid/L.

tive methods (vacuum filtration or decantation) of the original must taken as a control and the corresponding rosé wines. The musts were fermented to produced the corresponding rosé wines, which were stabilized and aged for a year in bottle. Prefermentative treatments were done in a wine cellar, and the fermentation was done in a pilot plant to simulate industrial conditions.

MATERIALS AND METHODS

Materials. *Vitis vinifera* var. *garnacha* must, Navarra Denomination of Origin, was collected; rosé wines were produced in a pilot plant. Must was inoculated with 0.5 g/L of Fermivin active dry *Saccharomyces cerevisiae* from Gist-brocades (F. Lafford and Cía SA, Pasajes, Spain). Reagents used were Merck analytical quality with controlled heavy metal content. The metal standard solutions (1000 mg/L) were obtained from Merck (Darmstadt, Germany).

Instruments. A stainless Marzola Marzinox (Marrodan and Rezola SA, Logroño, Spain) crusher−stemmer equipped with a rubber roller was used to crush the grapes. One portion of must was filtered through a rotary vacuum filter, Espal V-20 (Temavinsa, Logroño, Spain), with a 6500 L measuring barrel equipped with a 4 hp shaking motor, a 40 hp vacuum pump and a 7.5 hp feed pump. The diatomaceous earth filter, with a maximum particle size of 52 μm, had a surface area of 30 m²/g and a filtration volume of 8 000−10 000 L/h. Vinification was carried out in stainless steel (AISI 316-18/8/2) vertical tanks. Tank dimensions were 0.76 m diameter, 1.1 m in height, and 400 L capacity.

Must turbidity was determined using a 18900 Hach turbidimeter (Hach Co., Loveland, CO) prepared for colored samples. A Metrohm Polarograph (Metrohm Ltd., Herisau, Switzerland) equipped with an HMDE, model 647 VA stand, was used and connected to a 646 VA processor and a 675 VA sampler changer. An Ag/AgCl electrode in 3 M KCl was used as reference electrode, the auxiliary electrode being platinum.

Methods. *Vinification.* Newly-cropped *V. vinifera* var. *garnacha* grapes were crushed and destemmed. The skins were not removed for 17 h. Must was later divided into three fractions. The first was treated with SO_2 (50 mg/L) but was not subjected to any prefermentation technique. The other two, following refrigeration to 10 °C and the addition of SO_2 (50 mg/L), were clarified by two different prefermentation treatments: decantation or filtration by a rotary vacuum filter. In decantation, the must remained in stainless steel tanks for 24 h before racking. Then, 400 L of each of the three musts were subjected to fermentation using *S. cerevisiae* (0.5 g/L) and controlled temperature. Average temperature was 17.7 °C and the standard deviation less than 2 °C. In all cases, the fermentation was continued until the concentration of reducing sugars fell below 2.5 g/L. Finally, wines were stabilized by refrigeration at −5 °C for one week and then filtered through a cellulose pad in a plate and frame filter and aged in bottle at ambient temperature in the absence of light for one year.

Preparation Of Samples. (1) Acid Digestion (Oehme and Lund, 1979). A 40 mL amount of sample, 4 mL of H_2SO_4, and 4 mL of H_2O_2 were brought to boiling point, and every time a darkening of the sample was observed, 4 mL of H_2O_2 was added until the solution remained transparent and clear for

at least 5 min. The residue was left to cool and was then diluted to 100 mL with distilled deionized water. Total metal content was determined by this method.

(2) Acidification to pH 2 (Daniele et al., 1989). An aliquot of sample was adjusted to pH 2 by HCl addition and left to stabilize for 24 h before measurement. Thus metal displaced at acidic pH was obtained.

(3) Dilution. An exact volume of sample was diluted to 25 mL with distilled deionized water. This provided labile metal.

Study of the Influence of V_s and t_d on the DPASV Signal Characteristics. For each treatment, sample volumes of 0.5, 1, 2, 3.5, and 5 mL were taken. These volumes were diluted to 25 mL with distilled water. Measurements of i_p and E_p were performed directly on these dilutions. For each volume, deposition times of 30, 60, 90, 120, 180, 240, and 300 s were used.

Quantitative Metal Determination. Quantitative determination for each sample (must or wine) subjected to all three treatments was performed in the following way. For total metal content determination (treatment 1), 10 mL of acid-digested sample was diluted to 25 mL with distilled deionized water. Acid-displaced metal (treatment 2) was measured by acidification of 5 mL of the sample to pH 2 and dilution to 20 mL with distilled deionized water. Labile metal (treatment 3) was analyzed by taking 5 mL of sample and diluting it to 25 mL with distilled deionized water.

Voltammetric Procedure. The following parameters were used for recording the differential pulse anodic stripping voltammograms: nitrogen deaeration, 5 min; deposition time: (a) for the study of DPASV signal, 30, 60, 90, 120, 180, 240, and 300 s; (b) for quantitative analysis, 30 s for treatments 1 and 2 and 300 s for treatment 3; rest period, 20s; deposition potential, −1150 mV; mercury drop area, 0.40 mm²; scan rate, 10 mV/s; and pulse amplitude, 50 mV. Quantification was carried out using the standard addition method (two additions per measurement).

RESULTS AND DISCUSSION

Characteristics of Musts and Wines. General parameters of musts and stabilized wines are shown in Table 1. The two clarification treatments studied reduced must turbidity with respect to the control (1460 NTU) with vacuum filtration achieving greater levels of clarification (66 NTU) than decantation (205 NTU). The treatments did not modify pH, reducing sugars, or ammonium nitrogen of the must (Ayestarán et al., 1995). However, wine produced from filtered must had higher volatile acidity (0.74 g of acetic acid/L) and a lower concentration of higher alcohols (99 mg/L) than the decanted sample (203 mg/L) and the control (241 mg/L) (Ancín et al., 1996a, b).

Influence of t_d and V_s on Voltammetric Signal Characteristics. Zinc was characterized as forming highly labile species, fundamentally in the wines produced, as demonstrated by the well-defined stripping peaks obtained in all of the samples, in agreement with the results of Arcos et al. (1993) in red wines with different degrees of aging. The peak intensity, in
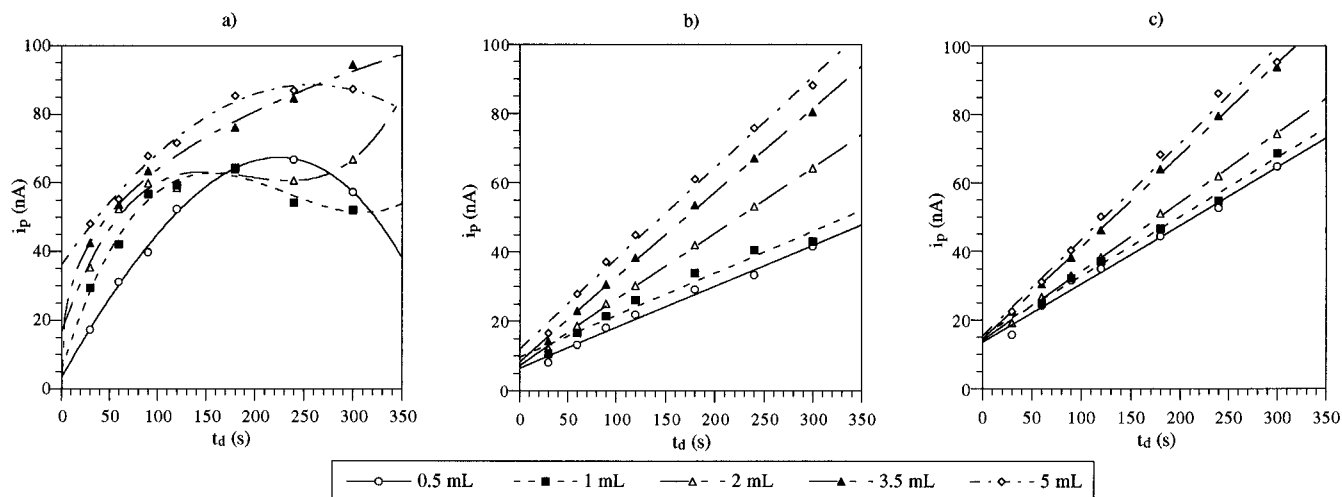
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Figure 1. Effect of deposition time and sample volume on the Zn stripping current (nA) for rosé wine from decanted must: (a) acid digested; (b) acidified to pH 2, (c) diluted.
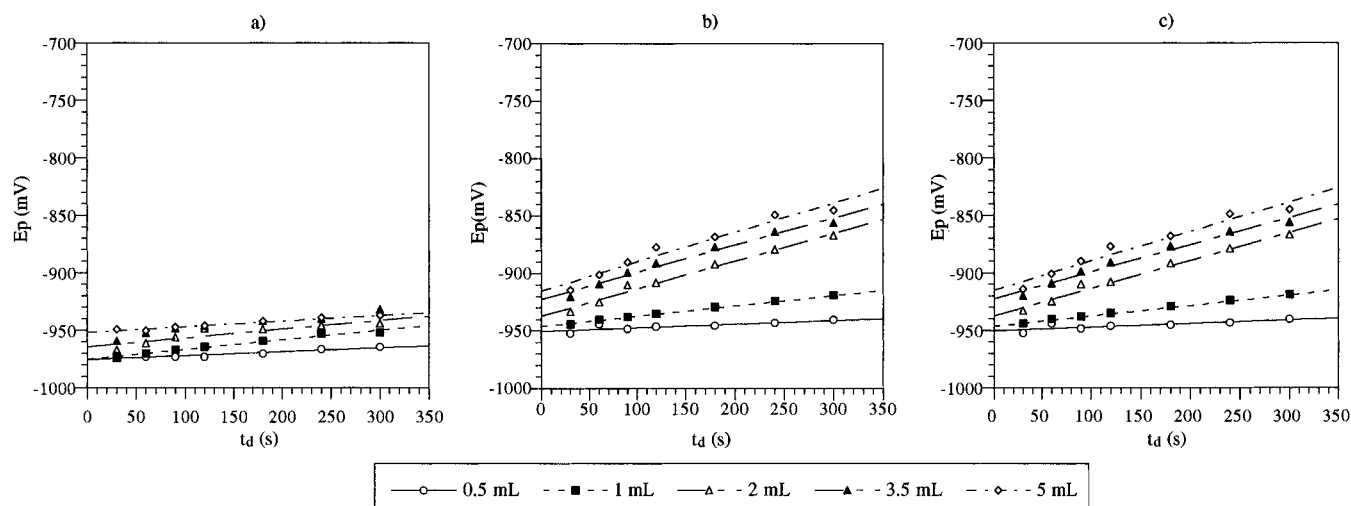

Figure 2. Effect of deposition time and sample volume on Zn stripping potential (mV) for filtered Garnacha must: (a) acid digested; (b) acidified to pH 2; (c) diluted.

general, in musts and wines (at natural pH and at pH 2) increased with the sample volume and with deposition time. As an example, parts b and c of Figure 1 represent the variation of i_p with t_d for zinc at different sample volumes of rosé wine produced from decanted must. However, in digested samples i_p increased with t_d until a maximum value was reached which varied depending on the sample volume taken (Figure 1a). This is due to interactions of metal with the residual matrix of the digestion medium that, given its acidic−oxidant character, could oxidize and dissolve part of the metal deposited on the mercury drop during the preconcentration process. In Figure 2 (filtered Garnacha must) it is apparent that stripping potential became more positive where the sample volume is increased. For small sample volumes (less than 2 mL) at pH 2 and for the diluted sample (Figure 2b,c), E_p varied little with deposition time, meaning that at these volumes organic material scarcely interfered with the electrode. In the case of the digested sample (Figure 2a), E_p had values more negative than in previous cases, confirming the hypothesis of acid−oxidant matrix interaction (Arcos et al., 1993).

Cadmium only exhibited stripping peaks in digested musts and digested wine produced from control and decanted musts. However, it was not detected at natural

pH nor at pH 2, so it should be strongly complexed by the organic and inorganic material present.

The labile lead species that gave a voltammetric signal at natural pH could be observed only in wines, had wide peaks, and the potential was more negative than expected. Similarly, at pH 2 stripping peaks were detected only in wines, were narrower and the intensity increased with t_d and V_s. In this case the potential was less negative and hardly varied with time or sample volume suggesting that at pH 2 the adsorption of organic material on the electrode was irrelevant. In digested samples, stripping peaks appeared in all cases, E_p varied little with t_d and V_s, but the peak intensity, i_p, increased linearly with deposition time and with the sample volume. The latter is shown in Figure 3 for the three wines. Therefore, it seems that lead in the musts formed complexes or was retained strongly since it was not possible to detect it even with acidification to pH 2. In the wines, with much lower amounts of organic material than the musts, lead also appeared to be forming stable complexes but in lesser proportions.

For copper peaks, i_p at V_s of 0.5 mL in the pH 2 sample and in the dilute sample appeared only with deposition times greater than 120 s, demonstrating that copper formed slightly labile compounds in the musts and wines studied. This can be observed, for example,
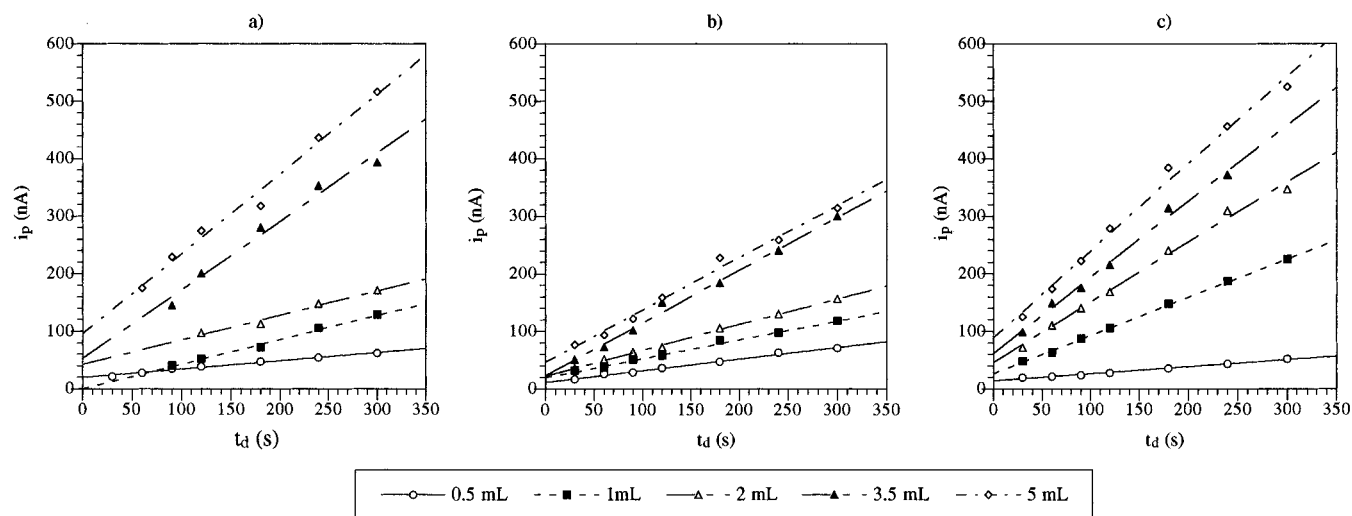
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Figure 3. Effect of deposition time and digested-sample volume on Pb stripping current (nA) for three wines from musts: (a) control; (b) decanted; (c) filtered.
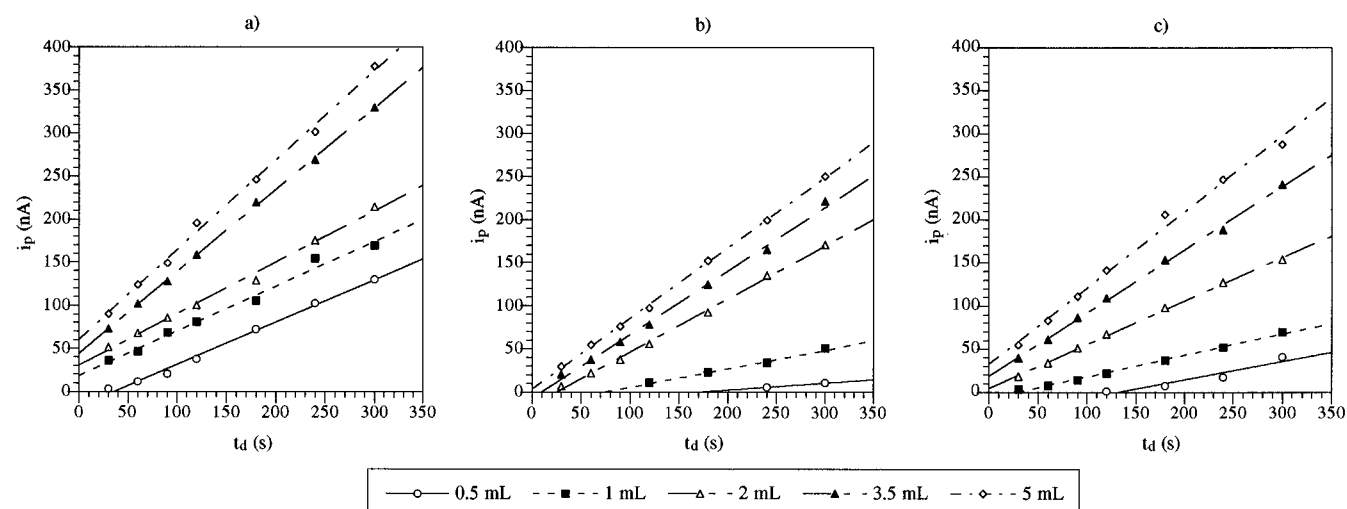

Figure 4. Effect of deposition time and sample volume on Cu stripping current (nA) for filtered Garnacha must: (a) acid digested; (b) acidified to pH 2; (c) diluted.

Table 2. Concentration of Zn, Cd, Pb, and Cu Obtained by DPASV of Garnacha Musts with Different Clarification Treatments: After Digestion, Acidification at pH 2, and Dilution ($n = 4$; All Parameters with Their Standard Error, SE)

		heavy metals (μg/L $\pm$ SE)			
must	sample preparation	Zn	Cd	Pb	Cu
control	digestion[b]	1053 $\pm$ 46	11 $\pm$ 2	1114 $\pm$ 51	1038 $\pm$ 42
	pH 2[c]	632 $\pm$ 23	a	a	683 $\pm$ 24
	dilution[d]	566 $\pm$ 5	a	a	483 $\pm$ 19
decanted	digestion[b]	769 $\pm$ 7	2.7$\pm$0.1	794 $\pm$ 67	1077 $\pm$ 23
	pH 2[c]	638 $\pm$ 15	a	a	688 $\pm$ 12
	dilution[d]	365 $\pm$ 27	a	a	427 $\pm$ 19
filtered	digestion[b]	603 $\pm$ 30	1.8 $\pm$ 0.1	204 $\pm$ 3	623 $\pm$ 65
	pH 2[c]	432 $\pm$ 3	a	a	577 $\pm$ 19
	dilution[d]	415 $\pm$6	a	a	437 $\pm$ 12

[a] Not detected. [b] Total metal. [c] Acid-displaced metal. [d] Labile metal species.

in Figure 4b,c, where the variation of i_p vs t_d for pH 2 and dilute samples of filtered must is shown for different sample volumes. In digested samples, i_p increased with sample volume and a variation proportional with deposition time was observed (Figure 4a).

Influence of Clarification on Metal Content of Must. In Table 2 concentrations of Zn, Cd, Pb, and Cu of Garnacha musts are listed corresponding to total (digested sample), acid-displaceable (pH 2 sample), and

labile (diluted sample) metal. In this table, it is observed that the control must has the highest total metal concentrations for all the cations except for copper, which had a similar value in the decanted and control musts. The clarification processes studied (filtration and sedimentation) diminished this content. Probably, this is due to both clarification processes eliminating protein nitrogen (Ayestarán et al., 1995) and colloidal material from the must dragging along the metals

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united with them. In general, the concentration of labile and of acid-displaceable metal decreased in the clarified musts with respect to the control allowing the conclusion that the clarification processes, besides eliminating strongly complexed species (not displaced with acid), also removed labile metal.

In regard to the influence of must sedimentation on total metal content, it is observed that this treatment did not eliminate copper but did eliminate zinc (26.9%), lead (28.7%), and a greater percentage of cadmium (75%). Vacuum filtration of must was more effective in decreasing the concentration of these cations than sedimentation: zinc (42.7%), lead (81.7%), and cadmium (83.6%). Copper was not removed by sedimentation, but it was eliminated by filtration (40%). Tromp and Klerk (1988) found that filtration of must reduced the concentration of copper, and Gorinstein et al. (1984) confirmed that copper, as well as iron, forms complexes with proteins. Ayestarán et al. (1995) observed that vacuum filtration of must eliminated a greater percentage of protein nitrogen (86.1%) than sedimentation (13.9%); all of this seems to confirm the existence of these complexes and their elimination during filtration. Similarly, the filter's retention of solids such as the remains of skins, the preferred location for oxidases, contributes also to copper elimination since it is associated with these enzymes (Amati, 1984).

Effective elimination of zinc, especially in the filtered sample (Table 2), with other factors, could have influenced the slower kinetics of fermentation shown for this must (Ancín et al., 1996b). Zinc is a growth factor for yeast, accelerates cellular reproduction, reduces total acidity, and increases the alcoholic degree (Fernández-Pereira, 1988). Schisler et al. (1982) established that fermentation kinetics are related to the content of certain nutrients present in the suspended solids, among which is zinc.

The origin of lead and cadmium in must, according to Eschnauer (1982), is secondary and, therefore, does not arise from the grape itself but from contaminations and/or adulterations; possible potential sources are agrochemical treatments, atmospheric pollution, products employed during vinification, or the very machinery used in the elaboration (Golimowski et al., 1979a,b; Eschnauer, 1982). Both elements have little importance from an enological point of view; however, they are important for their toxicological aspect (Gargano and Renon, 1983; Gavioli et al., 1984). The clarification treatments studied eliminated lead from the medium, filtration to a greater extent than sedimentation. Lead that remains in the medium must form very stable complexes which cannot be measured at pH 2 (Table 2). Cadmium was found in minor concentrations in the musts, and it was the most sensitive element to the clarification process, which practically eliminated it from the medium.

In summary, vacuum filtration processes diminished Zn and Cu, total metal as well as labile and acid-displaceable species. In filtered musts the percentage of labile Zn and Cu increased with respect to its total. It seems that sedimentation did not eliminate metal forms which are displaceable with acids at pH 2 since these concentrations were the same as the control must. Both metals of elevated toxicity, Cd and Pb, were greatly eliminated by the clarification process, and what remained in the must formed complexes of elevated stability that were not measurable by DPASV.

Table 3. Concentration of Zn, Cd, Pb, and Cu Obtained by DPASV of Wines from Garnacha Musts with Different Clarification Treatments: After Digestion, Acidification at pH 2, and Dilution (n = 4; All Parameters with Their Standard Error, SE)

wine from must	sample preparation	heavy metals (μg/L±SE)			
		Zn	Cd	Pb	Cu
control	digestion[b]	507 ± 27	2.9 ± 0.2	149 ± 7	137 ± 5
	pH 2[c]	427 ± 4	a	58 ± 4	65 ± 2
	dilution[d]	404 ± 16	a	47 ± 4	32 ± 1
decanted	digestion[b]	465 ± 10	2.4 ± 0.1	126 ± 6	118 ± 8
	pH 2[c]	358 ± 8	a	55 ± 1	29 ± 2
	dilution[d]	356 ± 4	a	43 ± 2	23 ± 2
filtered	digestion[b]	549 ± 20	a	115 ± 8	112 ± 8
	pH 2[c]	511 ± 14	a	51 ± 1	52 ± 4
	dilution[d]	416 ± 9	a	35 ± 2	16 ± 2

[a] Not detected. [b] Total metal. [c] Acid-displaced metal. [d] Labile metal species.

Metal Content of Stabilized and Aged Wines. Table 3 contains the results from the analysis of total, acid-displaceable, and labile metal for wines stabilized and aged for a year in bottle. From the data of Tables 2 and 3, it is evident that the wine elaboration process considerably reduced total metal and even completely eliminated cadmium from the wine produced from filtered must. During the fermentation process, the concentration of these metal ions decreases (González-Larraina, 1988) due to microorganism consumption, pH changes, alcoholic strength increase, redox reactions, and the time-induced evolution of the colloidal micelles. The reduction of total copper content in the wine produced from filtered must (82%) was somewhat less than in the wines produced from musts with more turbidity (decanted, 89%, and control, 86.8%). These results agree with those of Hsia et al. (1975), who observed greater elimination of copper in fermentation of musts with large amounts of suspended solids. Some solids are a sulfur source for yeast which reduce it to sulfide favoring the precipitation of copper sulfide, a compound with a very low solubility product. Similarly, Brun and Hamelle (1975) explained the disappearance of copper during fermentation as a process based on absorption and adsorption on yeast.

In each wine, due to elimination and/or modification of organic material during fermentation, the proportion of labile zinc with respect to total zinc was greater than in the must (Tables 2 and 3); this metal formed mostly labile species in wine (between 76% and 80%), and the percentages of these species did not differ much from the corresponding acid-displaceable species (between 77% and 93%). This cation principally formed easily disassociated compounds, an observation similar to that of Arcos et al. (1993). The proportion of labile metal with respect to total metal oscillated between 30.4% in wine produced from filtered must and 34% in wine produced from decanted must. In the wines, the concentrations of labile copper species were inferior to the starting musts (Tables 2 and 3), and the proportion of labile copper with respect to total copper was less in the wines than in the musts. This behavior was different to that described for zinc and lead since, in spite of having eliminated organic material during fermentation and stabilization, the percentage of labile copper was less. Lastly, the labile fraction of Cd was not detected in the wines produced from control or decanted must; this metal, present in low concentration, must form stable species with ions and ligands presents in the medium. In wine produced from filtered must,

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this cation was eliminated totally during the fermentation and stabilization processes.

The concentration of acid-displaceable copper and zinc was less in the wine than in the corresponding must with the exception of the wine produced from filtered must. Only acid-displaceable lead only was detected in wine, whereas acid-displaceable cadmium was not found in either must or wine (Tables 2 and 3). The difference between total metal and acid-displaceable metal corresponds to that which forms the strongest unions with the ligands present in the medium. In this sense, cadmium was found to be the most strongly complexed (100%), followed by copper and lead (both between 53% and 76%) and, finally, zinc (between 7% and 23%). This implies a real reduction in the potential toxicity of Cd and Pb since toxicity is a direct consequence of the most labile metal forms.

ABBREVIATIONS USED

DPASV, differential pulse anodic stripping voltammetry; E_p, peak potential; i_p, peak current; t_d, deposition time; V_s, sample volume; NTU, nephelometric turbidity unit.

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