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Characterization of the Proteic and the Phenolic Fraction in Tartaric Sediments from Wines*

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ABSTRACT

The proteic and phenolic fraction precipitated along with potassium bitartrate crystals during the different stages of Spanish sparkling wine-making were studied. Electrophoretic and chromatographic techniques, together with chemical analysis, were carried out to determine the nature and physico-chemical properties of the organic compounds present in sediments. The phenolic compounds were more abundant and highly polymerized in the samples obtained from cold stabilization. Cinnamic acids esterified with tartaric acid comprised the main low molecular weight phenolic compounds in all the samples. There was a propensity for certain proteins to precipitate out of the wines, given that the main bands detected in the tartrates presented higher electrophoretic mobilities than those in the wines themselves. The distribution of the molecular weights of the proteins in the tartrates differed from that of the proteins in the wines. The molecular weights of the main bands obtained using SDS-PAGE were smaller than 20 000 or higher than 50 000 in the case of the proteins from the tartrates, whereas the molecular weights of the main bands for the proteins in the musts and in the wines ranged between 20 000 and 40 000.

INTRODUCTION

The study of the behaviour of potassium bitartrate (KHT) in hydroalcoholic solutions and in wines has shown that precipitation is slower and non-

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stoichiometric in wines. This is because of selective adsorption of certain substances in the wines onto the faces of the crystals, thus impeding crystal growth (Rodríguez-Clemente & Correa-Gorospe, 1988; Rodriguez-Clemente *et al.*, 1990).

Koch & Sajak (1959) detected the presence of proteins in tartrate deposits, and Pilone & Berg (1965) and Balakian & Berg (1968) confirmed the important role of non-dialyzable compounds in the precipitation of KHT. In a recently published paper (Correa-Gorospe *et al.* 1991) the presence of nitrogenous compounds and polyphenolic compounds of varying degrees of polymerization has been demonstrated in tartrates from containers used for the fermentation, storage, and stabilization of wines used in the production of Spanish sparkling wines ('cavas').

Better knowledge of the compounds that precipitate along with KHT and inhibit the growth of KHT crystals and of whether the proportion of such compounds is the same as in the source wines or whether there is a propensity of certain of these compounds to precipitate out will, in turn, make possible improvements in wine stabilization techniques, thereby improving yields.

MATERIALS AND METHODS

Samples

The tartrates studied were collected (in an industry making sparkling wines) from containers used to hold white wines; the sources of the samples are set

TABLE 1
Origin of the Tartrates Analyzed

Sample no.	*Source description*
1	Tartrates deposited in stainless steel (AISI 316) tanks used for the storage of white wines.
2	Tartrates deposited in tanks of reinforced concrete lined with ceramic tiles used for raw white wines.
3	Tartrates from various sources collected during drying.
4	Tartrates from oak casks containing white wines of more than one vintage.
5	Tartrates deposited in stainless steel tanks used for fermenting and storing white wines.
6	Tartrates deposited in stainless steel tanks in which precipitation of KHT had been induced by storing white wines at −4°C for 10–12 days.
7	Tartrates deposited in lined concrete tanks in which precipitation of KHT had been induced by storing white wines at −4°C for 10–12 days.

out in Table 1. The musts and varietal wines analyzed were from the grape varieties Macabeo, Xarello, and Parellada used in the production of sparkling wines in Spain and collected in the same cellar as the tartrates.

Dissolution of the tartrates

The tartrates were ground to a fine, homogeneous powder in an agate mortar and dissolved in the proportion of 10 g/litre 0·1 N HCl.

Low-polymer phenols

Precipitation of the high-polymer compounds present in the samples was done in a saturated solution of sodium chloride (Masquelier *et al.*, 1965), followed by centrifugation, and determination of the low polymer phenols in the supernatant.

Leucoanthocyanins

These were determined by reaction with *n*-butanol:HCl (1:1) in a hot water bath at 100°C and measurement of optical density at 550 nm (Peri & Pompei, 1971).

Catechins

Reaction was with vanillin in a hydrochloric acid medium and measurement of absorbance at 500 nm (Rebelein, 1965).

Phenolic acids and phenolic acid esters by high-performance liquid chromatography (HPLC)

Chromatographic samples were prepared in accordance with the procedure described by Diez *et al.* (1980): 250 ml of samples of tartrates dissolved in 0·1 N HCl were concentrated, evaporated at 40°C, and the phenolic acids were extracted with ethyl ether, evaporated to dryness and the residue was taken up in 1 ml of methanol:water (1:1). A Waters liquid chromatograph consisting of two M-6000 A pumps, a M-720 solvent programmer, a M-U6K injector, a Novapak column (30 cm × 0·39 cm i.d.) with a particle size of 4 μm, and a M-440 UV detector with 280-nm and 340-nm filters was used. The chromatographic conditions were as stated in Fig. 1 (Fernandez de Simón *et al.*, 1990).

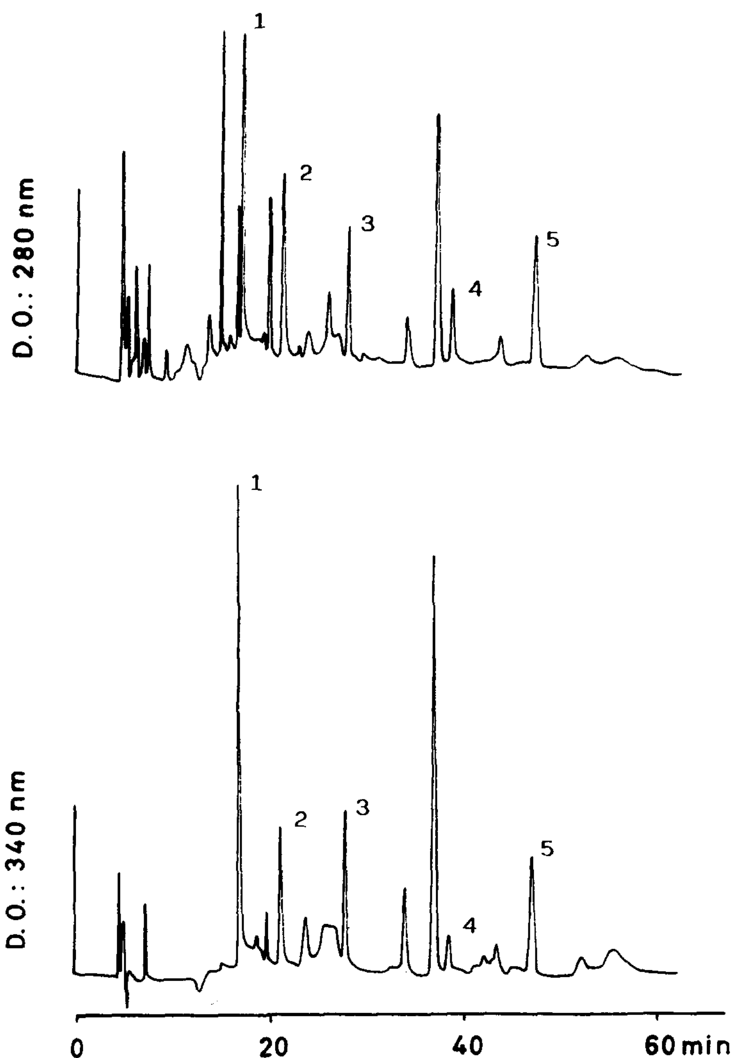

Fig. 1. Chromatogram of the low molecular weight phenolic fraction from tartrate sample 3 (origin listed in Table 1). Chromatographic conditions: Novapak column (30 cm × 3·9 mm i.d.), 4 μm. Solvent A: acetic acid/water (2/98); Solvent B: acetic acid/methanol/water (2/30/68). Gradient: 0 min (0% B, 0·6 ml min^{-1}); 10 min (40% B, 0·6 ml min^{-1}); 15 min (50% B, 0·6 ml min^{-1}); 20 min (60% B; 0·5 ml min^{-1}); 30 min (70% B; 0·4 ml min^{-1}); 40 min (75% B; 0·4 ml min^{-1}); 45 min (86% B; 0·3 ml min^{-1}); 50 min (85% B; 0·7 ml min^{-1}); 60 min (85% B; 0·8 ml min^{-1}). Curve 5. Peaks 1: caffeoyl tartaric acid; 2: *p*-coumaric tartaric acid; 3: feruloyl tartaric acid; 4: *p*-coumaric acid; 5: ferulic acid.

Preparation of samples for analysis of the protein fraction

The samples were dialyzed against tap water using membranes with a pore size of 3500 Daltons (Spectrum Medical Industries, Los Angeles, CA, USA) for 48 h. The dialyzates were concentrated by reverse osmosis by submerging dialysis bags in a 20% solution of Carbowax 20M (Merck, Darmstadt, FRG). The final concentration of the tartrates and the musts was 50 times, that of the wines 150 times, the source material.

Total protein

Reaction was with Coomassie Brilliant Blue G-250 (Bradford, 1976) of the concentrated dialyzed samples; calibration was carried out using bovine serum albumin (BSA) (Merck, Darmstadt, FRG).

Polyacrylamide gel electrophoresis (PAGE) without dissociating agents

The method of Hillier (1976) was employed using slabs measuring 170 × 130 × 0·7 mm. The slabs were stained with Coomassie Brilliant Blue G-250 according to the method of Blakesley & Boezi (1977).

Polyacrylamide gel electrophoresis with dissociating agents (SDS-PAGE)

The method employed was that of Laemmli (1970) with a stacking gel concentration composition of $T = 5\%$, $C = 1{\cdot}4\%$ and a resolving gel composition of $T = 10\%$, $C = 2{\cdot}7\%$. Samples were denatured by heating to 100°C for 5 min following Hames (1981). Molecular weights were calibrated using a kit by Pharmacia Fine Chemicals (Pharmacia LKB, Uppsala, Sweden) covering the range of molecular weights between 14 000 and 94 000 Daltons. The stain used was Coomassie Brilliant Blue R-250 according to the method of Hames (1981).

Isoelectric focusing (IEF)

A Multiphor M-2117 apparatus and a Multitemp II LKB M-2219 thermostatic circulator (Pharmacia LKB, Uppsala, Sweden) were used. The support was a polyacrylamide gel slab measuring 55 × 40 × 0·25 mm with a composition of $T = 7{\cdot}5\%$, $C = 3\%$ prepared at our laboratory according to the method described by Alonso & Gascó (1987) and a pH gradient of from 3·5 to 9·5. The conditions employed were: prefocusing at 0·7 W, 15 mA, and 300 V for 30 min, followed by placement of the sample on the gel and focusing at 1·2 W, 15 mA, and 700 V for 80 min; power was constant and the temperature 5°C in both stages. The pH was measured at 1-cm intervals on

the surface of the gel using a Multiphore Electrode M-2117-111 LKB surface electrode (Pharmacia LKB, Uppsala, Sweden) calibrated at 5°C. The stain was Coomassie Brilliant Blue applied according to the method of Blakesley & Boezi (1977).

Quantification of stain proteins

The densitometric measures were carried out at 600 nm with a Shidmadzu equipment composed of a spectrophotometer (Chromato Scanner CS-930) and an integration and graphic impression system (Data Recorder DR-2).

RESULTS AND DISCUSSION

Analysis of the phenolic fraction

Analyses of the phenolic compounds present in these same samples of tartrates and summarized in a previous paper (Correa-Gorospe *et al.*, in press) have shown that the concentration of such compounds was greater in the tartrates that had precipitated during cold stabilization.

The percentage ratio of low polymer phenols to total phenols was from 75 to 97 in samples 1 to 5 corresponding to tartrates from containers used for the fermentation and storage of the wines. In samples of tartrates from containers used for cold stabilization of the wines, samples 6 and 7, the percentage ratios were 16 and 20, respectively. No catechins were detected but small quantities of leucoanthocyanins were present.

Analysis of the low molecular weight phenolic fraction in the tartrates was carried out using HPLC. Figure 1 presents the chromatogram corresponding to sample 3. The analysis indicated that, in the samples of precipitates from cold stabilization, fermentation, and storage of the wines, the phenolic fraction consisted primarily of cinnamic acids (caffeic, *p*-coumaric and ferulic acids), mostly esterified with tartaric acid. In a study of the low molecular weight phenolic compounds in wines made from these same grape varieties carried out by Diez *et al.* (1984), no correspondingly large share of cinnamic acids was reported. Precipitation of the cinnamic acids may be induced by a combination of the presence of tartaric acid esterifying the cinnamic acids and by the tendency of the tartaric acids to take part in the formation of crystals of tartaric salts. The role of the tartaric acid that esterifies the phenolic acids as inhibitors of KHT and CaT precipitation should not be overlooked, even though its concentration in the wine may be quite small.

Characterization of the protein fraction

In a previous paper dealing with these same tartrates (Correa-Gorospe *et al.*, 1991), the tartrates were found to contain 0·011–0·064% protein (expressed as BSA), and the mean percentage ratio of protein nitrogen to total nitrogen was 3·53. The samples collected from containers used for cold stabilization contained the largest quantities of protein. The concentration of proteins in six musts of the grape varieties studied herein ranged between 3·7 and 9·7 mg of BSA/litre. The concentration of proteins in wines made from these same grape varieties was lower, from 1·6 to 1·8 mg of BSA/litre, among other reasons because of alcohol-induced insolubilization of the proteins and the deproteinizing treatments applied to the wines. The ratio of protein nitrogen to total nitrogen in these samples of must and wine was less than 1% in the musts and less than 0·2% in the wines. Although the tartrates analyzed did not come from these same wines precisely, concentration (or enrichment) of proteins in the tartrates can be inferred, because wine-making procedures are highly standardized and the composition of the wines of different vintages is also quite similar (Polo *et al.*, 1983; Cabezudo *et al.*, 1986; Martin-Alvarez *et al.*, 1987).

Polyacrylamide gel electrophoresis of the samples of tartrates from containers used for the fermentation and storage of the wines (samples 1 to 5), without the use of dissociating agents, revealed only a single band that

TABLE 2

Percentage Distribution of Bands Obtained using PAGE and Stained with Coomassie Brilliant Blue G-250 for Tartrates, Musts and Wines of Grape Varieties used in the Spanish Sparkling Wine-Making

Samples	*Electrophoretic mobility*											
	0·28	*0·32*	*0·35*	*0·39*	*0·42*	*0·45*	*0·50*	*0·52*	*0·55*	*0·58*	*0·60*	*1·00*
TARTRATES												
No. 6	—	—	1·5	6·6	—	12·7	9·6	8·6	—	5·4	—	55·6
No. 7	—	—	2·6	7·3	—	9·2	13·2	6·9	—	5·5	—	55·3
MUSTS												
Parellada	—	—	14·8	77·4	—	4·9	2·9	—	—	—	—	—
Macabeo	—	—	13·6	26·2	29·2	19·1	11·5	—	—	—	—	—
Xarello	—	—	40·4	23·1	11·3	15·2	3·9	2·3	0·4	3·4	—	—
WINES												
Parellada	3·2	17·7	—	26·1	—	24·2	19·1	6·4	—	—	—	3·3
Macabeo	3·1	4·6	—	11·6	—	30·8	34·2	12·1	—	—	—	3·6
Xarello	3·8	7·7	—	15·5	2·7	27·0	27·3	—	9·4	—	0·7	5·9

advanced along with the tracking dye; in other words, it had a high charge: molecular weight ratio, at the pH at which the electrophoresis was carried out. There were, in addition to this band, a further six bands with mobilities of between 0·35 and 0·58 in the tartrates from containers used for cold stabilization (samples 6 and 7) (Table 2).

From four to seven bands with mobilities of between 0·35 and 0·58 were detected in the musts made from the grape varieties used in this study. This range of mobilities was similar to that reported for musts made from different grape varieties (Correa *et al.*, 1988; Gonzalez-Lara *et al.*, 1989; Polo *et al.*, 1990). Bands with mobilities of between 0·28 and 0·60 were detected in the wines. Some of the bands detected in the musts were not detected in the wines, which instead presented bands with higher and lower mobilities than the bands in the musts.

SDS-PAGE carried out on the tartrates yielded bands with molecular weights ranging from 10 000 to more than 80 000 (Table 3). The bands were clustered in two regions, one for molecular weights of between 10 000 and 20 000 and the other for molecular weights of between 60 000 and 80 000. There were no appreciable differences between the electrophoretograms for

TABLE 3
Percentage Distribution of Bands obtained using SDS-PAGE and Stained with Coomassie Brilliant Blue R-250 for Tartrates, Musts and Wines of Grape Varieties used in the Manufacture of Sparkling Wines

Samples	*Molecular weights (Daltons × 10^{-3})*							
	>80	*79–70*	*69–60*	*59–50*	*49–40*	*39–30*	*29–20*	*19–10*
TARTRATES								
1	—	—	51·3	12·5	—	—	—	33·9
2	—	—	73·4	—	—	—	—	26·5
3	—	—	100·0	—	—	—	—	—
4	35·4	18·2	12·5	16·1	—	—	—	17·4
5	—	45·9	23·5	—	—	30·6	—	—
6	—	15·1	23·7	—	—	—	41·0	19·7
7	—	8·2	13·1	—	10·0	25·2	—	43·0
MUSTS								
Parellada	—	—	5·4	5·5	—	28·2	51·8	9·1
Macabeo	5·2	—	8·9	4·0	2·3	—	77·5	1·6
Xarello	4·3	—	6·5	—	—	17·3	62·9	9·0
WINES								
Parellada	5·2	—	9·4	—	2·6	31·8	45·8	4·8
Macabeo	5·6	—	8·5	—	—	40·1	45·6	—
Xarello	—	—	8·3	—	—	32·6	59·1	—

the tartrates collected from the different sources, which contrasted with the results of the electrophoretic analysis in which dissociating agents were not used.

The range of molecular weights of the bands for the musts and the wines made from the grape varieties used, obtained using SDS-PAGE (Table 3), was, like that for the tartrates, from 10 000 to more than 80 000, but the molecular weights of the main bands were between 20 000 and 40 000. These results agreed with those reported for musts (Correa *et al.*, 1988; Gonzalez-Lara *et al.*, 1989; Polo *et al.*, 1990) and for wines (Hsu & Heatherbell, 1987; Laminkara & Inyang, 1988) made from other grape varieties. The range of molecular weights of the main proteins was very similar to that reported by Dubordieu *et al.* (1986), Interesse *et al.* (1987), and Polo *et al.* (1990) using HPLC and by Moretti & Berg (1965) using ultracentrifugation. The fact that the molecular weights of the main proteins in the tartrates were higher than those of the main proteins in the musts and in the wines indicates that there was a tendency for the more highly polymerized proteins to be adsorbed by the crystals.

The range of isoelectric points (pI) for the proteins in the tartrates

TABLE 4
Percentage Distribution of Bands obtained using Isoelectric Focusing and Stained with Coomassie Brilliant Blue G-250 for Tartrates, Musts and Wines of Grape Varieties used for Spanish Sparkling Wine-Making

Samples	*Isoelectric points*							
	7·5–7·1	*6·6–6·4*	*5·7–5·5*	*5·4–5·2*	*5·1–4·9*	*4·8–4·6*	*4·5–4·3*	*4·2–4·0*
TARTRATES								
1	—	—	—	—	17·9	39·0	43·8	—
2	—	—	8·3	6·3	52·5	21·1	7·1	4·7
3	—	—	9·9	—	45·9	23·4	12·2	8·6
4	—	—	15·2	—	41·7	21·7	—	21·4
5	—	—	—	—	31·8	36·9	31·3	—
6	—	—	3·4	1·8	37·9	46·6	9·3	—
7	—	—	—	—	34·6	58·6	6·8	—
MUSTS								
Parellada	3·5	—	1·6	6·1	4·8	37·8	16·0	33·6
Macabeo	—	—	15·8	3·7	3·6	33·0	43·7	—
Xarello	1·7	—	—	4·5	6·2	33·2	53·6	0·9
WINES								
Parellada	4·1	—	—	12·9	67·7	1·8	11·0	—
Xarello	3·8	2·6	—	8·5	4·8	64·9	6·1	7·5
Macabeo	—	—	—	—	92·7	—	7·3	—

(Table 4) was between 4·0 and 5·7, while those for the main proteins in the wines ranged between 4·3 and 5·1. No appreciable differences were recorded among the pI values for the proteins from the various tartrate samples nor among the isoelectric points for the main proteins from the tartrates, the musts, and the wines.

CONCLUSIONS

Phenolic compounds present in tartrates collected from containers used for cold stabilization of wines were more highly polymerized than those present in tartrates collected from containers used for fermentation and storage of wines. Cinnamic acids esterified with tartaric acid comprised the main low molecular weight phenolic compounds in all the samples. There was a propensity for certain proteins to precipitate out of the wines, given that the main bands detected in the tartrates presented higher charge densities, than those in the wines themselves. The molecular weights of the main bands obtained using SDS-PAGE were smaller than 20 000 or higher than 50 000 in the case of the proteins from the tartrates, whereas the molecular weights of the main bands for the proteins in the musts and in the wines ranged between 20 000 and 40 000. The pI of the main proteins in tartrates, musts and wines ranged between 4·3 and 5·1.

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