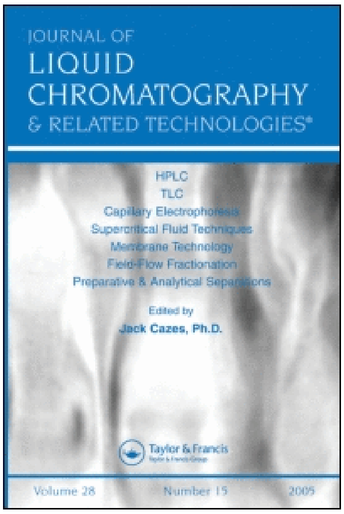

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DETERMINATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF ORGANIC ACIDS IN SPANISH ROSÉ WINES FROM THE ALPUJARRA-CONTRAVIESA REGION OF GRANADA

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ABSTRACT

High performance liquid chromatography was used to analyze the principal organic acids present in wine (tartaric, malic and lactic acids) after purification of samples with Sep-Pak C_{18} cartridges (Waters). The method was applied to 40 samples of home-made wines from the Alpujarra-Contraviesa region of the province of Granada (Southeast Spain). These wines are considered rosé, but in most cases had been subjected to unintentional maceration. The results obtained showed that the wines had not undergone complete malolactic fermentation and presented low concentrations of tartaric acid. The proportion of the concentrations of malic and lactic acids suggested a possible alteration by the undesirable development of lactic bacteria.

INTRODUCTION

Three organic substances of an acid character (tartaric, malic and lactic acid) are present in wine must and normally remain in the wine in lower concentrations.

Other acids also found in wines are galacturonic acid, in concentrations from 0.04 to 1 g/l, glucuronic acid (0 - 0.6 g/l) and oxalic acid (0 - 60 mg/l) (19).

Yeast metabolisms produce lactic, succinic and acetic acids (26), whose concentrations and individual characteristics are important, in addition to the acidity common to all three.

Tartaric acid is the most abundant of all and the most acidic in the must and wine. It originates in the grapes, where it forms in the stem, skin and pulp (25) from the sugars of the young shoots or from oxalacetic acid.

The malic acid content increases in the grapes, where it forms from 3-phosphoglyceric acid during growth up to a concentration of 15-20 g/l (34). However, during ripening this level falls considerably as a result of respiratory combustion and transformation of the acid into glucides, which takes place in the ripe grapes by a process inverse to the carboxylation of piruvic phosphoenol acid (26), (13), in which temperature is the predominant factor.

In the wine, the malic acid is transformed into either ethanol or lactic acid and carbonic anhydride by *Schyzosaccharomyces pombe*. For this reason only small quantities of this acid are found in finished wines, in which the smoother taste of lactic acid improves the sensory characteristics (9). However, volatile acidity is increased by simultaneous decomposition of citric acid (16), which raises the pH, thus favouring the development of microbial agents (30) (14) (19).

In recent years, many papers have been published on acid determination by liquid chromatography, such as those by Bevilacqua & Califano (5); Romero *et al.* (27) and López *et al.* (17).

There are also several bibliographic studies such as those by Barcenilla (4); Matas (20) and Polo *et al.* (24), which consider different protocols. Some workers use ion-exchange chromatography with strongly acid cationic resins and strongly or weakly basic resins. Symons (33) applied this technique to the determination of non-aromatic and phenolic acids, prior to which the sample was passed through a resin precolumn connected to an anion-exchange column; detection was by refractometry. Frayne (10) developed a method for determination of acids together with fructose, glycerine and ethanol in musts and grapes using a double ion-exchange system with parallel UV and infrared detectors.

Other workers, such as Antolin & Lugar (2) or Schneider *et al.* (31) used a reverse phase with C8 or C18 columns and buffer solutions with pH < 3 as elutents; separation based on the difference in hydrosolubility.

According to these papers, detection can be carried out by differential refractometry (31) (32) (33) at UV or other wavelengths close to 210 nm (214 nm Rousseau *et al.* (28), 220 nm Antolin & Lugar (2), 212 nm Auguste & Bertrand (3) or 206 nm Shaw & Wilson (32)).

The main problem in preparation of the sample is that it must be pure enough not to present interferences. Some authors therefore consider direct injection to be suitable for white wines (2) (3), whereas for red wines other authors recommend the use of active carbon (3), Sep Pak C_{18} cartridges (2) (28), or an anion (31) or cation (33) (28) exchange resin. Other workers (32) propose a mixed method.

Different columns have also been proposed for determination of these acids by HPLC. Schneider *et al.* (31) used reverse phase with Aminex HPX87-H column after passing the sample through Sep Pak C_{18} cartridge. Antolin & Lugar (2) used a Lichrosorb RP-18 reverse phase column; Blanco *et al.* (6) examined apple juice and ciders for these acids using a Lichrosorb RP-18 (C18) column; Marce (18) studied the acids in fruit juices and wines using a precolumn filled with Bondapak C18/Corasil; García-Jares *et al.* (11) used a Spherisorb C-8 reverse phase column after passing the sample through Dowex 2 anion-exchange resin.

The aim of this paper is to provide significant data on the content of the principal organic acids present in the wines produced in the Contraviesa region of the Alpujarra near Granada (Spain), which has a long viticultural tradition. The factors which led us to determine the most relevant physical-chemical parameters of these rosé wines, including the organic acids, are the basically homemade nature of production, the retail distribution and the lack of analytic control. The organic acids provide information leading to characterization and typification of the wines, as well as information on malolactic fermentation and their state of preservation (bacterial alterations).

MATERIALS AND METHODS

Reverse phase high performance liquid chromatography, using a variable wavelength UV/VIS detector, was applied to determination of organic (tartaric, malic and lactic) acids in 40 samples of wine produced in the Alpujarra-Contraviesa region.

1. Experimental conditions

HPLC instrumentation consisted of a Konik model KNK-500A chromatograph, with a Konik injector model 7125. The column used was a Lichrospher Merck CH-18, 10 cms long, interior diameter 4.7 mm, 5 μm internal particle size. The mobile phase consisted of an aqueous solution of 2% H_3PO_4, with 1 ml/min flow rate. A Konik spectrophotometric detector model UVIS 200 was used with 220 nm wavelength. The experiments were carried out at 40°C and the injection volume was 10 μl.

2. Reactives

Standard solutions of organic acids obtained by dissolution of the different acids (MERCK) in a hydroalcoholic mixture (13% (v/v) ethanol) with 0.75-12 g/l acid concentration range.

2% KH_2PO_4 aqueous solution with pH adjusted to 2.2 by H_3PO_4.

3. Preparation of samples

In order to eliminate polyphenolic substances the rosé wine samples were passed through a Sep Pak C_{18} (Waters) cartridge previously solvated with ethanol and then filtered through a 0.45 μm Millipore membrane.

4. Analytic technique

Figure 1 shows the chromatogram corresponding to a standard solution of organic acids.

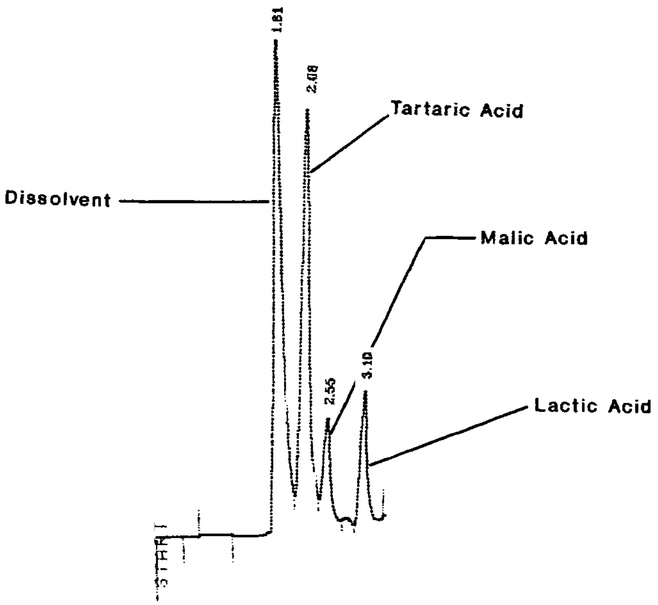

FIGURE 1. Chromatogram of a standard solution of organic acids in hydroalcoholic solution at 13% (v/v).

Identification of the components was carried out according to their retention times. These were determined by injecting the sample 10 times into the chromatograph. The results are shown in Table 1.

Quantification was carried out by calculating the equation of the calibration lines obtained from the standard solutions. The statistical results are shown in Table 2.

5. Validation of the method.

We studied the reproducibility of the method on the basis of 10 injections of a 13% (v/v) hydroalcoholic mixture containing 3 g/l, 1.5 g/l and 1.5 g/l concentrations of tartaric, malic and lactic acid respectively, under identical experimental conditions. The results are shown in Table 3.

TABLE 1. Identification of components (retention times).

ACID	RETENTION TIME (min.)	RSD (%)
Tartaric	2.08	0.6
Malic	2.55	1.4
Lactic	3.10	1.2

TABLE 2. Statistics of calibration lines.

	Tartaric Ac.	Malic Ac.	Lactic Ac.
Maximun	885156	463855	226407
Minimun	53816	27750	16435
99% Conf.	390384.6	204489.5	98216.0
A	-2903.96	494.08	10058.66
b	73964.59	38743.41	18530.65
R	0.999997	0.999987	0.995809

TABLE 3. Study of the reproducibility of the method.

Acid	Mean (Area)	S_{n-1}	RSD(%)
Tartaric	218273	2336.7	1.07
Malic	58109	552.0	0.95
Lactic	70886	778.9	1.09

Validation of the analytic technique proposed for determination of non-volatile organic acids by HPLC was made by the addition method. In order to verify the accuracy of the determinations made on a wine sample, known quantities of each of the organic acids were added to the sample. The results are shown in Table 4. Accuracy is expressed as a recovery percentage.

RESULTS AND DISCUSSION

According to the results obtained, statistical study of which is shown in Table 5, the tartaric acid levels (Fig. 2) are mainly low, with values of 1.00 to 2.68 g/l and a mean concentration of 1.70 g/l.

TABLE 4. Recovery percentages obtained in accuracy analysis.

ACID	Initial Amount (g/l)	Aded (g/l)	Found (g/l)	Recovery (%)
Tartaric	0.94	0,80	1.58	90.4
	0.94	1.20	1.97	92.0
Malic	0.46	0.80	1.14	90,5
	0.46	3.22	3.24	88.2
Lactic	1.33	1.30	2.50	95.0
	1.33	5.20	6.12	93.7

TABLE 5. Statistical analysis of results.

Acid	n	Mean(g/l)	S_{n-1}	RSD(%)	RANGE
Tartaric	40	1.7	0.41	24.1	1.0-2.7
Malic	40	1.3	0.29	23.0	0.8-1.8
Lactic	40	1.5	0.89	61.0	0 -3.1

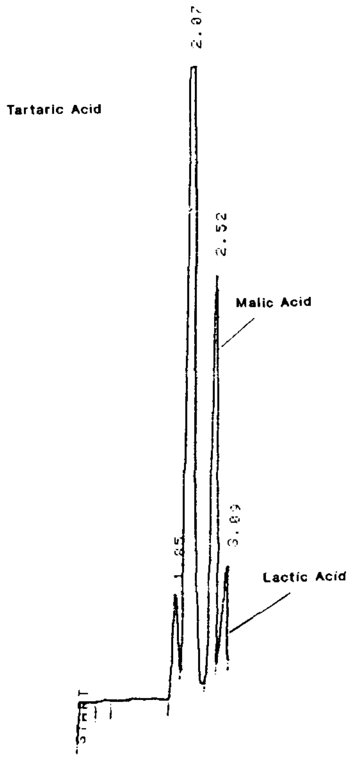

FIGURE 2. Chromatogram of a wine sample.

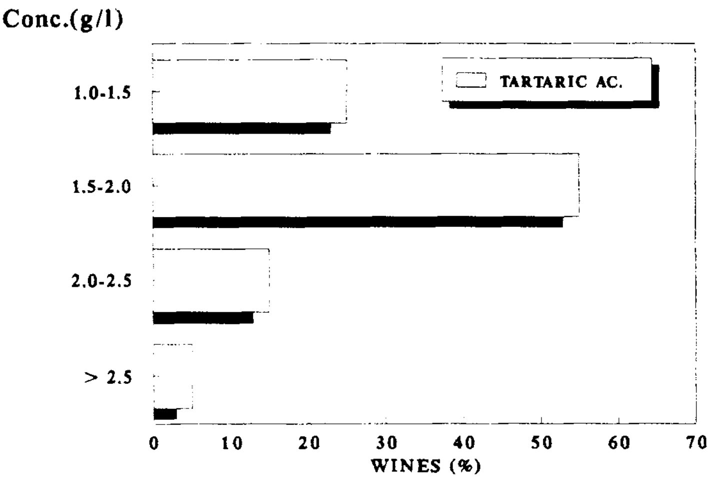

FIGURE 3. Distribution of tartaric acid (g/l) in the wines studied.

TABLE 6. Reference values in literature.

COUNTRY	REGION	TYPE	TARTARIC AC. (g/l)		REFERENCE
			MEAN	RANGE	
GENERAL			---	1,5-4,0	MARECA, J. (19)
GENERAL			---	2,8-8,0	AMERINE,M.A.(1)
SPAIN	*Galicia*		2,1	1,1-3,6	GARCIA,C.M. (11)
SPAIN	*Rioja*	Red	2,55	--------	SANTOS,M. (29)
			2,03	--------	
		White	1,66	--------	
			2,57	--------	
FRANCE			----	1,5-4,0	RIBEREAU,J.(26)
GERMANY			----	1,0-3,0	REBELEIN,E. (25)
ITALY			1,6	0,96-2,61	MODI,G. (21)
SPAIN		"Chacolí"	2,99	2,03-4,22	CAMPO, G. (7)
SPAIN	*General*	White	2,63	1,18-4,08	GOROSTIZA,E.F(12)
		Red	1,65	0,82-2,6	

This determines a tartaric acid profile for these wines similar to that of others such as Galician (11), Rioja (30) and some Italian wines (21). In addition, these values agree rather well with the homemade character of the wines, as they are exposed to spontaneous tartaric precipitation (they are not subjected to any stabilizing process) which reduces both the acid and the potassium contents (8).

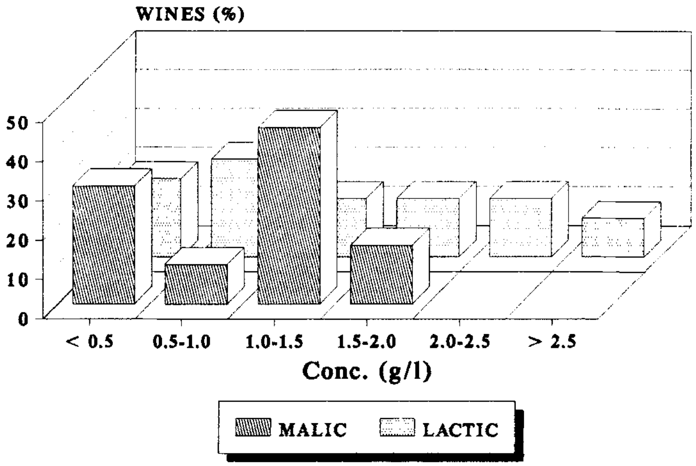

FIGURE 4. Distribution of malic and lactic acid (g/l) in the wines studied.

TABLE 7. Reference values in literature (continued).

COUNTRY	REGION	TYPE	MALIC AC.(g/l)		LACTIC AC.(g/l)		REFERENCE
			MEAN	RANGE	MEAN	RANGE	
GENERAL			----	0-3	---	---	MARECA, J. (19)
SPAIN	*Galicia*		0.53	0.016-1.6	---	---	GARCIA,C.M. (11)
SPAIN	*Rioja*	Red	trace	----	1.13	---	SANTOS,M. (29)
			----	----	2.06	---	
		White	1.93	----	1.94	---	
			----	----	0.49	---	
GERMANY			----	5-8	----	0-5	REBELEIN,E. (25)
ITALY			0.41	0.06-0.75	1.16	0.48-3.56	MODI,G. (21)
SPAIN		"Chacolí"	1.9	0.2-4.0	----	0.1-3.4	CAMPO, G. (7)
SPAIN	*Navarra*	Red	----	0.1-0.4	----	1.8-3.0	DIEZ DE BETANCOURT, C.A. (9)
		White	----	0.6-1.7	----	1.0-5.3	
	Levante	Red	----	0.0-2.0	----	0.2-2.5	
		White	----	0.2-1.0	----	0.5-1.5	
	Galicia	White	0.1	0.1-1.0	3.21	-------	IÑIGO,B. (15)
		Red and Rosé	0.8	0.2-3.5	2.6	-------	

With the exception of twelve wines containing only traces of malic acid and which, therefore, have undergone malolactic fermentation, the malic acid content (Fig. 3) in the remainder varies from 0.8 g/l to 1.8 g/l. Most of these wines have a medium to high level of malic acid in comparison with data found in the literature (Table 6), particularly in the case of wines such as clarets (wines subjected to slight maceration with the solid parts of the grape).

The lactic acid levels (Fig. 4) (1.5 g/l average value) mainly fall within the normal limits found in the literature (Table 7). However, Herranz & Mareca (14) suggested that if the concentration of lactic acid is higher than 0.6 g/l and that of malic acid higher than 1 g/l, which is the case of 35% of the wines studied here, or higher than 1 g/l and 0.4 g/l respectively (10% of the wines studied), there is reason to suspect alteration by development of undesirable lactic bacteria. Our results on the proportions of malic and lactic acids imply the existence of this type of alteration, which agrees with the high values of volatile acidity found in these wines (23).

CONCLUSIONS

High performance liquid chromatography of samples previously purified with Sep-Pak C_{18} (Waters) cartridges proves to be a rapid, reliable method for determination of the main organic acids present.

In general, these are wines with low levels of tartaric acid (they are subjected to spontaneous tartaric precipitation as they do not undergo any previous stabilization process). Not all of them undergo malolactic fermentation and, judging by the proportional concentrations of malic and lactic acids, there is even the possibility of alteration by development of undesirable lactic bacteria.

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