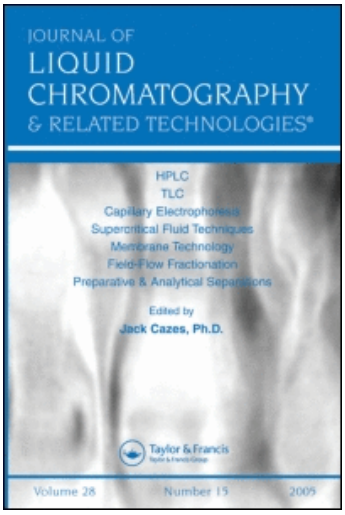

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PHENOLS HPLC ANALYSIS BY DIRECT INJECTION OF SHERRY WINE VINEGAR

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ABSTRACT

Analysis of sherry wine vinegars using reverse phase HPLC and diode array detection, via direct injection is performed. The technique proved to be useful to study phenolic composition, the identification of major peaks being achieved. The following compounds were detected in all the samples analyzed: gallic acid, caftaric acid, tyrosol, hydroxymethylfurfural and, in almost every sample analyzed, p-coumaroyltartaric glucosidic ester, caffeic acid, p-coumaric acid and caffeic ethyl ester. The results obtained may be helpful to establish a phenolic profile for sherry vinegars which may be used as an origin recognition pattern. These vinegars have been recently awarded their certification brand recognition.

INTRODUCTION

Due to the diversity of wine vinegars in the market and the increase in demand, it has been considered necessary to investigate reliable analytical methods to establish quality and origin criteria as the authentification remains unsolved. Among wine vinegars, sherry vinegars are specially recognized. Their elaboration from sherry wine and subsequent aging in oak barrels

following the 'solera' system gives singular organoleptic properties to the final product.

Polyphenolic compounds have been exhaustively reviewed in relation to origin, elaboration, aging and browning of wines.[1,2,3]

High Performance Liquid Chromatography has been employed for the study of phenols in vinegars.[4,5] Usually, the sample is subjected to a prior extraction before injection. However, substantial changes in sample composition may occur due to cis-trans isomerizations and hydrolysis phenomena occurring as a result of the extraction procedure.

To solve this problem in the analysis of phenolic compounds in wines, the direct injection of the wine onto the column with no other prior treatment but filtration was proposed.[6,7] These analytical methods allow a real and accurate knowledge of the sample, as no alteration in phenol composition occurs. Besides, the quantification is exact, as it avoids the losses associated with an extractive approach. However, this technique has not previously been applied to vinegars.

The characterization of phenolic compounds is enhanced by means of photodiode array detection, as it provides useful information related to the molecular structures.[8]

The aim of this work is to identify phenolic compounds of traditional sherry vinegars by means of HPLC with no handling of the sample, as well as the identification of other major peaks which remained unknown until now, in order to establish the whole phenolic profile of these vinegars. This point takes special interest since sherry vinegars have been recently awarded their certification brand recognition (appellation origin).

MATERIALS AND METHODS

Samples

The analyzed samples (n=22) are derived from different producers of Jerez wine vinegars and were purchased in local markets. The declared periods of aging were variable.

A wine vinegar sample obtained in an experimental bioreactor by quick

acetification process from sherry wine, with no subsequent aging, was also studied. It was included in this study for comparative purposes.

HPLC

The chromatograph employed was a Waters 600E system controller (Milford, Massachusetts, USA) connected to a Waters 996 photodiode array detector. Data treatment was performed with a Millenium 2.0 data station. The injection system is a syringe loading sample manual injector Model 7125 from Rheodyne (Cotati, California, USA). The temperature was controlled by a Waters Steel Column Heater Module.

The column was a Merck Superspher 100 RP-18 (250-4mm), particle size 4 μ, protected by a guard cartridge Nova-Pak C_{18} module from Waters. The volume injected was of 50 μL.

Chromatographic Conditions

The chromatographic conditions were originally described for the analysis of simple phenols and flavonols in wines.[6] Recently, the method was enhanced by changing the acetic content of the solvents used in the gradients.[7]

The vinegar was injected onto the column without any handling except for filtration with Millex-GV_{13} 0.22 μm filters.

The solvents:

A: acetic acid - water(1/99)
B: acetic acid - water (6/94)
C: acetic acid - water - acetonitrile (5/65/30)

The gradient profile was as follows:

Time	%A	%B	%C
0	100	0	0
15	0	100	0
30	0	100	0
50	0	90	10
60	0	80	20
80	0	70	30
120	0	0	100

The flow was 0.5 mL/min and the temperature was set at 22.5°C. Solvent is heated as it travels through the heater before entering the column. An internal cover maintains thermal stability during operation. The temperature stability within the column compartment is ±0.1 degree Celsius over the entire operating range.

Reagents

Simple phenols (gallic, caffeic, p-coumaric, ferulic...), catechin, vanillin, tyrosol and hydroxymetylfurfural (HMF) were purchased from Sigma (Milwaukee, USA), Carlo Erba (Milano, Italy), Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany). Standards were prepared in a concentration of 10 mg/L in water. The water employed was filtered through a Milli-Q system and the others solvents were furnished by Carlo Erba.

Hydroxycinnamic Ester Synthesis

Ethyl esters were synthetisized by the trifluorurum method. Two mL of trifluoruethane were added to a solution of 100 mg of acid in 50mL of absolute ethanol. After 48 hours, the amount of ester formed was enough for the identification.

Identification

Simple phenols were identified by matching their spectra with those of standards and by the comparison of their retention times with standards. In doubtful cases, the samples were spiked with standard and the purity of the peak obtained checked by comparison of spectra within the peak from peak liftoff to peak touchdown. Different shapes in overlayed spectra revealed any coelution. The Photodiode Array Detector together with the software uses a complex mathematical technique to automatically determine spectral matches of standard to sample spectra and compound purity testing. The overall shapes of spectra are converted into vectors in multidimensional space and then those vectors are compared. When a peak purity test is performed, the spectrum at the peak apex is the reference spectrum. All other spectral data contained within that peak are compared with the spectrum of the apex. Match results are reported in terms of angle between vectors' degrees. If two vectors (representing spectra) completely overlap, there is difference of zero degrees between them; therefore they represent the same compound.

The identification of mixed acids such as cafeoyltartaric acid, coumaroyltartaric, and the ester of glucose with this last one, was carried out using the spectra provided under the same conditions of the method described by Professor Dr. Roggero[7] (Laboratoire de Chimie Organique et Analytique, Faculte de Sciences de Avignon, France) to whom we are grateful.

Table 1

T_R, λ_{max}, of the Identified Peaks

Peak No.	T_R	λ_{max}	Identification
1	9.6	260	?
2	14.3	264	?
3	16.0	269	?
4	18.4	272	Gallic Acid
5	25.3	285	5-(Hydroxymethyl)-2-furaldehyde
6	26.8	262.3	?
7	27.8	312	Caffeoyl-tart acid
8	34.8	312	p-Coumaroyl-tart.ac caffeoyl glucosidic ester
9	37.6	314	p-Coutaric
10	38.0	278	2-Furaldehyde
11	43.6	278	Tyrosol
12	57.5	267	?
13	59.7	324	Caffeic Acid
14	69.4	276	?
15	70.4	271.7	?
16	86.2	309	?
17	89.5	310	p-Coumaric Acid
18	100.1	324	Ferulic Acid
19	108.1	255-355	Isoquercitrin
20	123.9	328	Caffeic Ethyl Ester
21	141.3	310	p-Coumaric Ethyl Ester

RESULTS AND DISCUSSION

More than 20 compounds have been identified in sherry vinegars. Their spectra have been recorded with the photodiode detector. Table 1 shows the retention time, the λmax value found and the identification of some peaks. Some of them have remained unidentified in sherry vinegars till now (HMF, furfural, tyrosol, cafeoyltartaric acid, p-coumaroyltartaric acid, isequercetrin).

Despite the complex chromatographic profile, as there are other compounds which absorb in the ultraviolet region besides the phenolic ones, it has been proved that the separation between peaks is adequate. Most of the peaks were shown to be pure by using the detector's peak purity test so that this technique is highly recommended for the study of phenolic compounds in sherry vinegars. The peak purity test is performed by taking spectra from peak apex through to the tail. The absorbance contribution of a second contribution compound, if any, reshapes the spectrum. It may be assumed that a peak which is spectrally homogeneous throughout the whole peak is a single and well separated compound.

Table 2 illustrates the compounds identified in the samples we analyzed. As can be observed, not all the compounds are present in every sample. However, there are some points in common that should help us to establish a phenolic pattern in sherry vinegars.

Among simple phenolic acids it can be noted that gallic acid is present in all the samples analyzed. p-Hydroxybenzoic and vanillic acids were not detected, although they have been reported by other authors in sherry wine.[9] Special attention was paid to protocathecuic acid which had been reported to be present in sherry vinegar (Galvez et al., 1993). A compound with very similar spectral characteristics (λ = 262, 293) was detected at a very close time to protocatechuic acid standard. When the samples were spiked with the standard, a unique peak was obtained, but the verification of purity (peak purity test) pointed out spectral inhomogeneity (Fig. 1).

Caffeic and p-coumaric acid were generally found in most sherry wine vinegars. On the other hand, ferulic was seldom identified. Cis-isomers of tartaric-hydroxycinnamoyl acids, whose retention times in reversed phase chromatography are slightly smaller than those of the *trans* compounds have been investigated without success. However, p-coumaroyl ethyl ester and caffeic ethyl ester have been properly identified.

Table 2

Compounds Identified in Sherry Wine Vinegars

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	+	+	+					+	+				
2	+	+	+	+		+	+	+					
3	+	+	+	+			+	+	+		+		
4	+	+	+	+	+	+	+			+	+	+	
5	+	+	+	+	+		+	+			+	+	+
6	+	+	+	+			+	+	+			+	+
7	+	+	+	+			+	+	+		+	+	
8	+	+	+				+	+	+				
9	+	+	+	+			+		+	+	+	+	+
10	+	+	+	+		+	+	+	+	+		+	+
11	+	+	+	+			+	+	+		+	+	+
12	+	+	+	+	+	+	+	+	+		+	+	+
13	+	+	+	+			+	+	+			+	+
14	+	+	+	+	+	+	+	+	+			+	+
15*	+	+	+	+			+	+	+			+	+
16	+	+	+	+			+	+					
17	+	+	+	+		+	+	+	+				
18	+	+	+	+	+	+	+	+	+			+	+
19	+	+	+	+	+	+	+	+	+			+	
20	+	+	+	+	+	+	+	+	+			+	+
21	+	+	+	+		+	+	+	+		+	+	
22	+	+	+	+		+	+	+	+			+	+

* Wine vinegar obtained in an experimental bioreactor

A: Gallic Acid; B: HMF; C: Cafaric Acid; D: p-Coumaric Glucoside Ester; E: p-Coumaric Acid; F: 2-Furaldehyde; G: Tyrosol; H: Caffeic Acid; I: p-Coumaric Acid; J: Ferulic Acid; K: Isoquercetrin; L: Caffeic Ethyl Ester; M: p-Coumaric Ethyl Ester.

With very few exceptions, the information provided according to the elaboration process is scarce. Besides, it was very difficult to obtain a large number of samples from different producers. Due to the above mentioned constraints, it was difficult to attribute the differences found either to the original subtract nor to the elaboration procedure.

As the caftaric acid is a substrate for the polyphenoloxidase and its

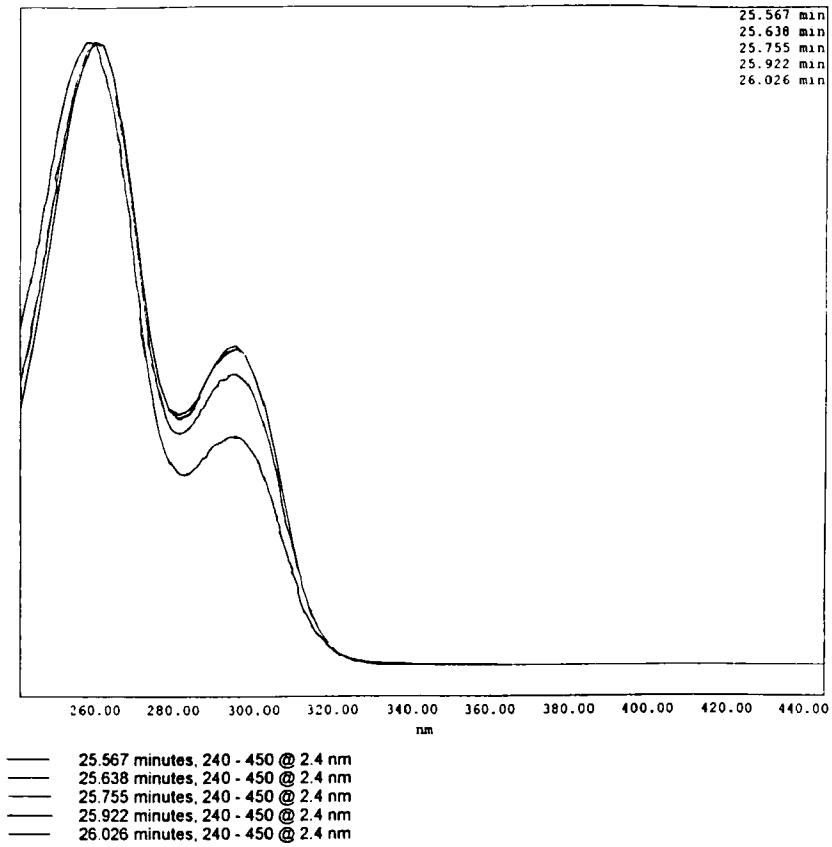

Figure 1. Spectra obtained by spiking the sample with protocatequic acid.

content in musts decreases during aging, and under oxidative conditions,[10] it is surprising to find this compound in almost every sherry vinegar analyzed, taking into account that vinegars are submitted to an oxidative medium and those of Jerez have suffered a browning process. This phenomenon should be explained by considering that a 'solera' is a dynamic system in which fresh wine is periodically added while a part of the most aged product is withdrawn and bottled. The presence of caftaric acid should be a useful indicator to differentiate sherry vinegars elaborated by a dynamic 'solera' system from those coming from a static one. Further research is required to confirm this point.

The largest peak in the 280 nm chromatogram is 5-hydroxymethylfurfural (HMF). Even before being identified, its value to differentiate sherry wine

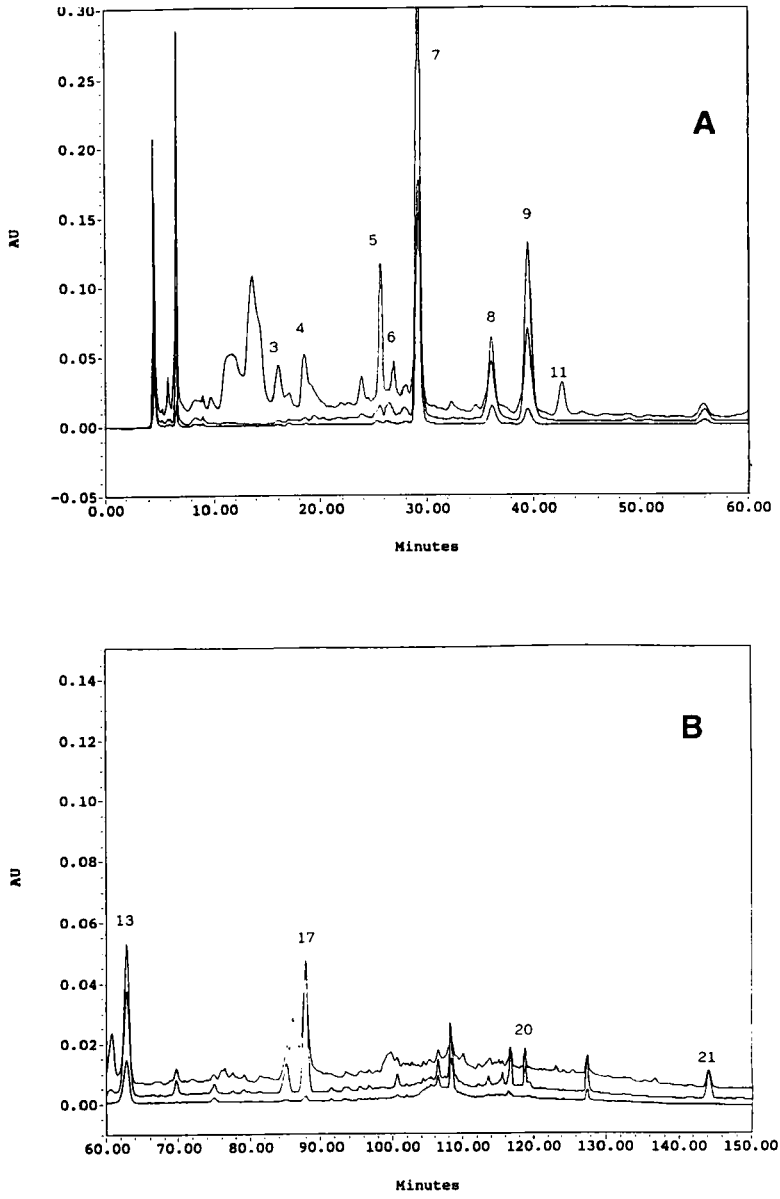

Figure 2 (a&b). Chromatogram corresponding to a sherry vinegar sample obtained by a quick acetification process.

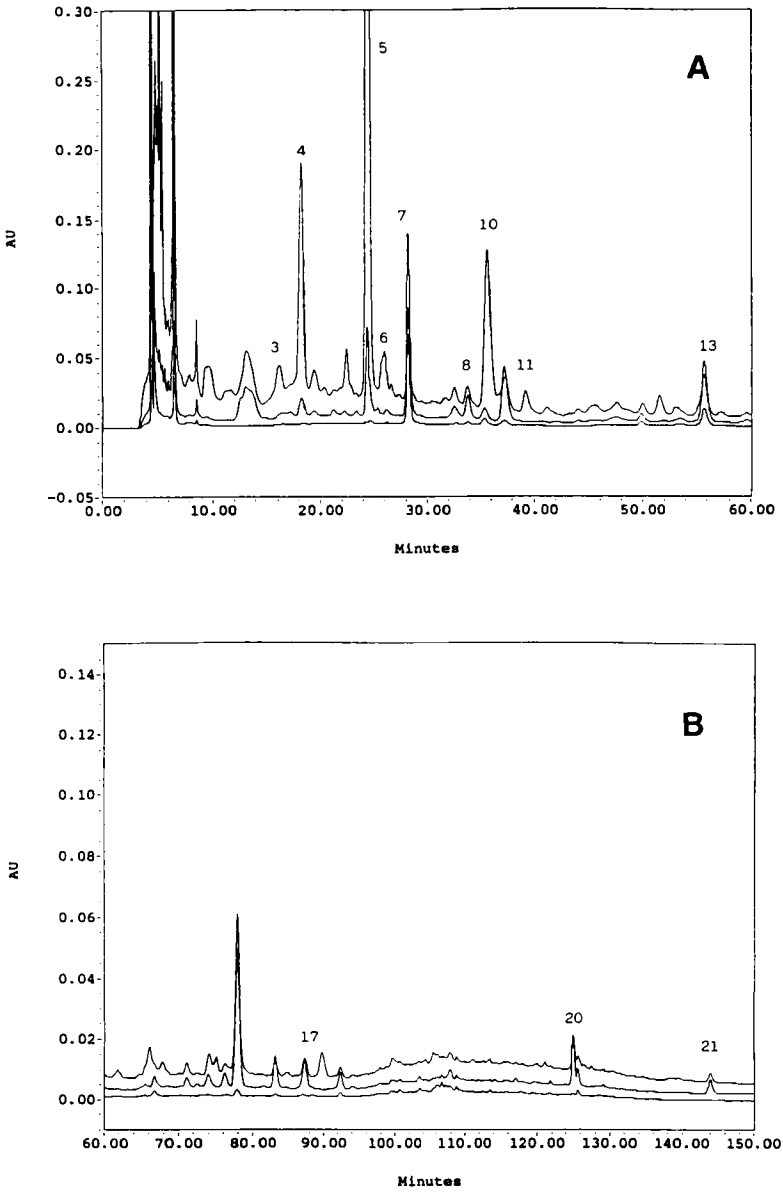

Figure 3 (a&b). Chromatogram corresponding to a sherry vinegar sample obtained by a"solera" system.

vinegars from those of other origin had been pointed out.[11]

Furfural and HMF have been included in the table despite their non-phenolic nature, due to the similiarity of their spectra with those of simple phenols.

HMF is barely detected in the sherry vinegar produced by a quick acetification process which has not been submitted to aging (Fig. 2), but its presence is very relevant in aged samples (Fig. 3).

Although these results are promising, the addition of must caramel requires one to be careful in drawing conclusions. This practice is well extended in vinegar wineries. Further research is necessary to prove the relationship of HMF to sherry vinegar's aging. But, it is clear that the absorbance at 280 nm proposed as measure of phenolic compounds in white wines[12] cannot be used as measure of phenolic compounds in sherry vinegars due to the enormous contribution of this compound to the global absorbance at 280 nm.

The utility of the technique to separate and characterize, not only the phenolic compounds, but also products of the Maillard reaction in sherry vinegars in a single run, has been proved. On the other hand, some compounds have been identified for the first time in vinegar and are essential to establish a fingerprint of sherry wine vinegar. Besides, there are others such as cafeoyltartaric acid and HMF which reveal differences in the elaboration process (aging and caramel addition). So that, the technique is of great interest to determine the origin, elaboration and aging of vinegars.

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