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Selection of column and gradient for the separation of polyphenols in sherry wine by high-performance liquid chromatography incorporating internal standards

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Abstract

A chromatographic column was selected, out of five possibilities tested, which offered sufficient efficiency to separate 21 out of 22 polyphenolic compounds, and the optimum elution gradient. Applied to samples of "fino" sherry wine, a satisfactory resolution of peaks was achieved, which allowed for the inclusion of an internal standard. The chromatographic behaviour of three polyphenolic standard substances was studied using the column and gradient selected, with a view to using them as internal standards for controlling the reliability of the analysis. It was concluded that all three can be used for this task, although 2,5-dihydroxybenzaldehyde is the most suitable in terms of ease of detection at 340 nm; however, the possibility of using the other two phenolic acids, β-resorcylic and 2,6-dimethyoxybenzoic acid, is not discarded as they could conveniently be made use of depending on the chromatographic profile of the sample to be analyzed.

Keywords: Wine; Polyphenols; Catechin; Epicatechin; Phenolic acids; Aldehydes

1. Introduction

The role that phenolic compounds play in the browning process of white wines has long been known [1]. This phenomenon, which in the case of "fino" sherry wine is known as "remontado", presents a great economic problem for wine cellars and it is important to have methods of analysis to determine the species implicated in this process. Wine samples are extremely complex and paper chromatography [2], which was originally used to analyse these compounds and other chromatographic methods have been replaced by HPLC owing to its superior resolution. Various methods for the determination of phenolic compounds in vegetable matrices using HPLC have been reported, including cocoa beans [3], soy beans [4] and especially grape, must and wine samples. Some optimized the elution by using the isocratic mode [5] and others by gradient elution [6–8], but nearly all workers have adopted reversed-phase C_{18} columns, eluting with mixtures of methanol–acetic acid–water or acetonitrile–water, with UV detection at wavelengths between 254 and 340 nm.

Although some workers have developed elu-

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tion gradients with a view to determining polyphenols in wine by direct injection into the HPLC system [9–11], this results in chromatograms which are too complex for reliable identification and quantification. On the other hand, in the case of sherry wine, the variability of concentrations of the different polyphenolic compounds which can be expected to occur is high so that it becomes necessary to resort to separation techniques for the samples in order to ensure that analytical signals which can be easily measured are obtained. Hence it is of interest to be able to control the quality and reliability of the analysis, which is feasible provided that one or more internal standards can be added at the beginning of the analytical process.

In this work, the chromatographic separation of 22 polyphenolic species was studied, with a view to being able to use various internal standards. For this purpose, five reversed-phase C_{18} columns of different lengths and with different particles sizes were tested using various gradients.

2. Experimental

2.1. Reagents and standards

The substances used as standards were obtained from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany) and Eastman Kodak (Rochester, NY, USA). Methanol of HPLC-gradient grade and all the other chemicals of analytical-reagent grade were purchased from Merck.

In all cases, the water used was of HPLC quality, purified in a Milli-Q system (Millipore, Bedford, MA, USA). All the prepared solutions were filtered through 0.45-μm membranes (Millipore) and degassed in an ultrasonic bath before being used.

The final preparation of the gradient was performed with a mixture of catechin, epicatechin, fourteen phenol acids and six aldehydes, at the concentrations given in Table 1.

Table 1
Composition of the mixed solution of standards

No.	Compound	Concentration (mg/l)
1	Gallic acid	21.2
2	Protocatechuic acid	27.0
3	Protocatechualdehyde	14.4
4	Gentisic acid	167.0
5	*p*-Hydroxybenzoic acid	36.6
6	Catechin	86.3
7	2,5-Dihydroxybenzaldehyde	84.1
8	*p*-Hydroxybenzaldehyde	9.6
9	β-Resorcylic acid	18.4
10	*m*-Hydroxybenzoic acid	29.0
11	Vanillic acid	12.1
12	Caffeic acid	12.2
13	2,6-Dimethoxybenzoic acid	33.2
14	Syringic acid	11.6
15	Vanillin	33.6
16	Epicatechin	90.5
17	Syringaldehyde	16.0
18	*p*-Coumaric acid	8.6
19	*o*-Vanillin	31.4
20	Ferulic acid	14.0
21	Veratric acid	67.8
22	Sinapic acid	31.4
23	*o*-Coumaric acid	7.6
24	3,4,5-Trimethoxycinammin acid	10.6
25	3,5-Dimethoxybenzaldehyde	26.0

2.2. Equipment

A Waters chromatograph fitted with two pumps, Models 510 and M-45, controlled by a Model 680 gradient programmer, a U6K injector and an M991 diode-array detector registering between 210 and 390 nm were used.

2.3. Columns

In all cases, the columns studied were steel cartridges with a C_{18} spherical particle filling, with the characteristics specified in Table 2.

2.4. Preparation of wine samples

The continuous rotary extraction process established previously [12] was used to obtain samples of the wine extracts to be injected. A

Table 2
Characteristics of the columns tested

Column	Type	Length × I.D. (mm)	Nominal particle size (μm)	Pore size (nm)	End-capped
A	LKB Spherisorb	100 × 4.0	3	10	No
B	LKB Spherisorb	250 × 4.0	5	10	No
C	LiChrospher	125 × 4.0	5	10	No
D	LiChrospher	250 × 4.0	5	10	No
E	Novapak	150 × 3.9	4	6	Yes

volume of 100 ml of water was added to 100 ml of wine and the resulting solution was saturated with NaCl. This solution was extracted with 80 ml of diethyl ether at a speed of 0.8 rpm for 3 h. The organic solvent was dried for 1 h with anhydrous sodium sulphate followed by evaporation in a rotary evaporator to attain a volume of about 0.5 ml, diluting to 5 ml with methanol–water (1:1) in a measuring flask.

3. Results and discussion

The extreme complexity of some wine samples resulted in low resolution of the chromatographic separation that we usually used for the analysis of phenolic compounds [13], thereby making impossible the identification and quantification of the sample composition and the introduction of an internal standard. This was the reason for seeking a column which would enable to achieve a suitable resolution of the samples being studied and would allow for the inclusion of the internal standard.

Bearing these considerations in mind, the solution to the problem was to search for a column offering the largest number of theoretical plates. To this end, we tested five reversed-phase C_{18} columns, selecting the one whose elution gradient resolved most of the species. In order to optimize the respective elution gradients, a mixture of 22 standards substances (catechin, epicatechin, phenolic acids and aldehydes) was used.

The methodology used to establish the elution gradient was the same in all cases: 20-μl samples of the solution of standards were eluted with a linear gradient made up from solvent A (10% methanol–2% acetic acid in water) to 50% of solvent B (90% methanol–2% acetic acid in water) in a period of 30 min at a flow-rate of 1 ml/min. Subsequently, the gradient and the concentrations of methanol in the initial phases were suitably modified to achieve the optimum separation which would make it possible to include an internal standard in the free spaces remaining between the peaks.

Finally, the best separations obtained for each of the columns tested yielded 21 peaks for columns C, D and E, 20 peaks for column B and 19 peaks for A. From a comparison of the five chromatograms, it could be clearly seen that the best separations are those achieved with columns D and E with their corresponding gradients (Fig. 1). In both situations, there are free spaces in the chromatogram in which at least one internal standard could be introduced. To make a choice, the chromatographic parameters [retention factor (k), resolution (R_s) and selectivity (α)] obtained for each species with these two columns and with the best conditions are presented in Fig. 2.

With respect to k, it can be observed that the distribution of peaks is more uniform in the case

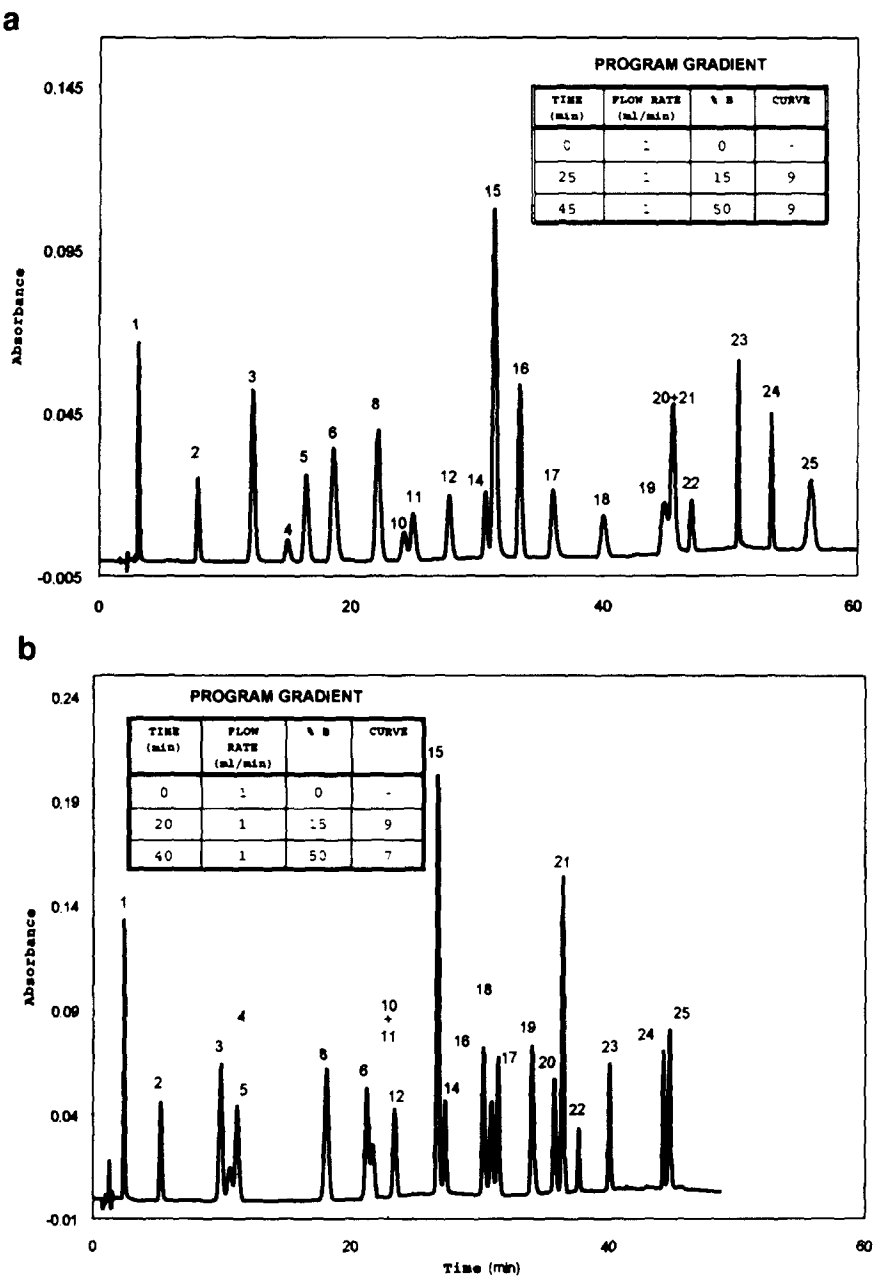

Fig. 1. Chromatograms obtained at 280 nm from a standard mixture of 22 polyphenolic compounds for best separations obtained with columns (a) D and (b) E and their respective phases of elution. For peak numbers, see Table 1.

of column D, and that although some large spaces exist with column E, there are also areas where partial overlap between peaks occurs.

Concerning the selectivity, it can be observed that there are no great differences, with variations ranging between 1 and 1.5 for nearly all the

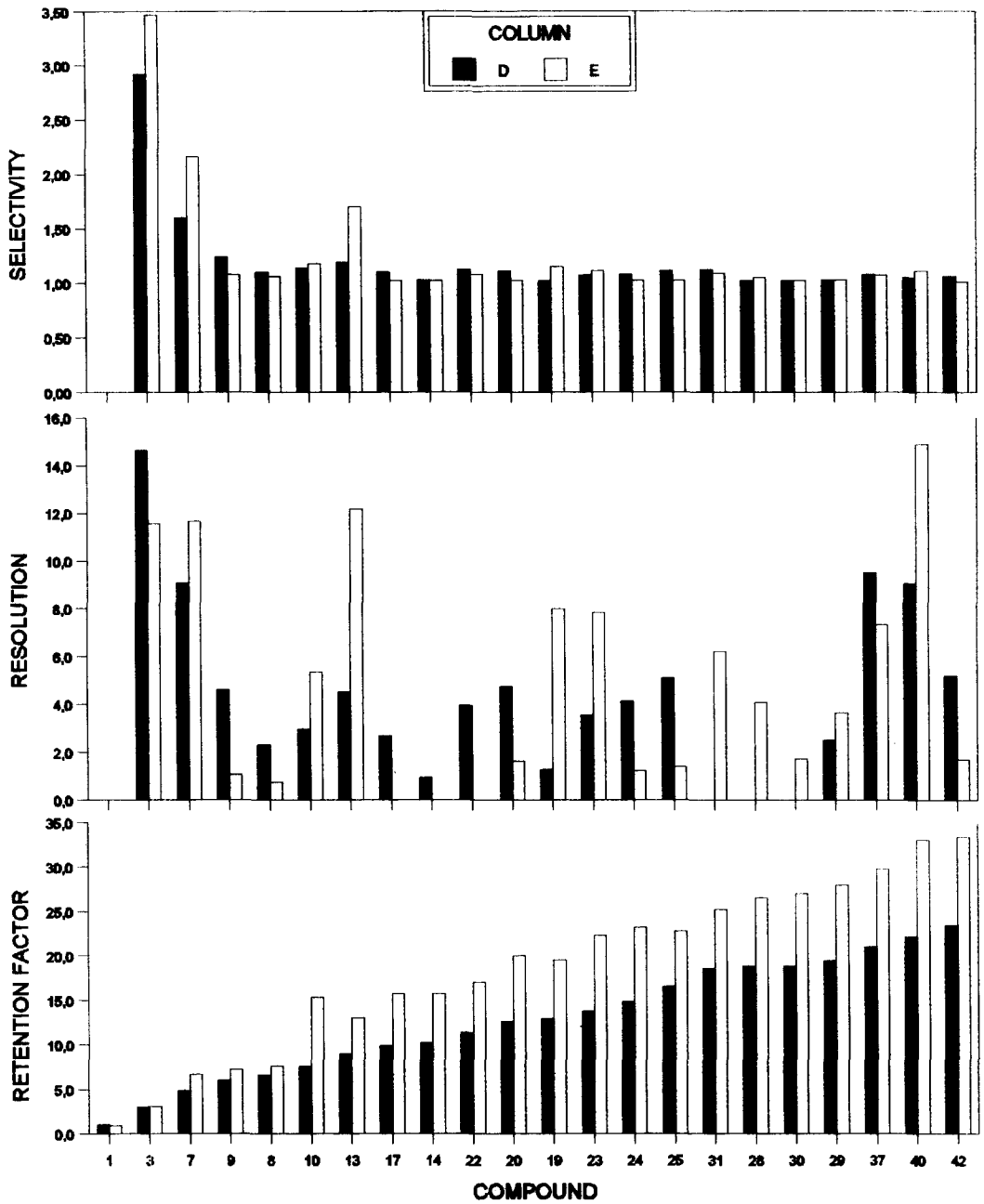

Fig. 2. Chromatographic parameters (k, R_s and α) for each species obtained with columns D and E under the optimun conditions. For peak numbers, see Table 1.

species; only column E, which is end-capped, shows a different selectivity at the beginning of the chromatogram.

Therefore, column D and the corresponding gradient (Fig. 1a) were selected to be applied to real samples. In Fig. 3, a chromatogram for a diethyl ether extract of a sample of "fino" sherry wine is shown. The peak resolution is acceptable. In addition, the chromatogram exhibits void zones potentially suitable for the possible inclusion of an internal standard.

For the selection of possible polyphenolic substances to be used as internal standards, we considered the collection of polyphenol standards existing in our laboratory, most of which have been studied in respect of their presence in or absence from wine and whose chromatographic behaviour is hence known.

Two phenolic acids (β-resorcylic and 2,6-dimethoxybenzoic acid) and one phenolic aldehyde (2,5-dihydroxybenzaldehyde) were tested. These were added successively to the mixture of standards which we had used to prepare the gradient and to extracts of wine and it was observed (Fig. 4) how the three standards tested could all be used for this task. 2,5-Dihydroxybenzaldehyde is perfectly usable since, although it overlaps somewhat with the *p*-hydroxybenzaldehyde, it is easily quantifiable at 340 nm, as it appears alone in a given area.

4. Conclusions

The use of a reversed-phase C_{18} column of 250×4 mm I.D. with a particle size of 5 μm, together with a two-stage gradient, permits the separation of 21 polyphenolic species with good resolution. The application to real samples, i.e., diethyl ether extracts of wine, involves the resolution of a large number of peaks which allows for the inclusion of internal standards. The best option of the three compounds investigated for their use as internal standards was 2,5-dihydroxybenzaldehyde, although the possibility of resorting to the other two compounds is not discarded, depending on the particular chromatographic profile, of the sample to be analysed.

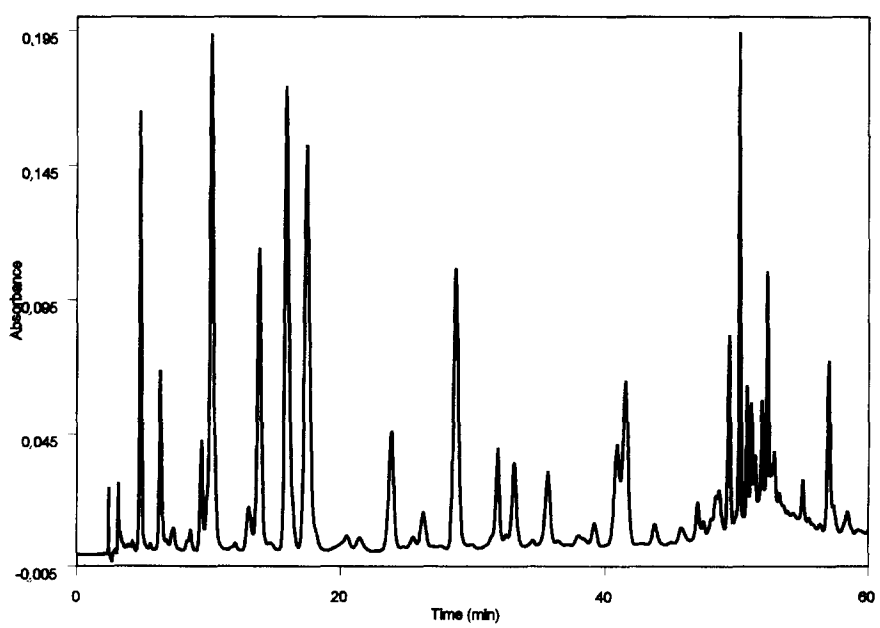

Fig. 3. Chromatogram obtained at 280 nm from a diethyl ether extract of wine using column D and the gradient described in Fig. 1a.

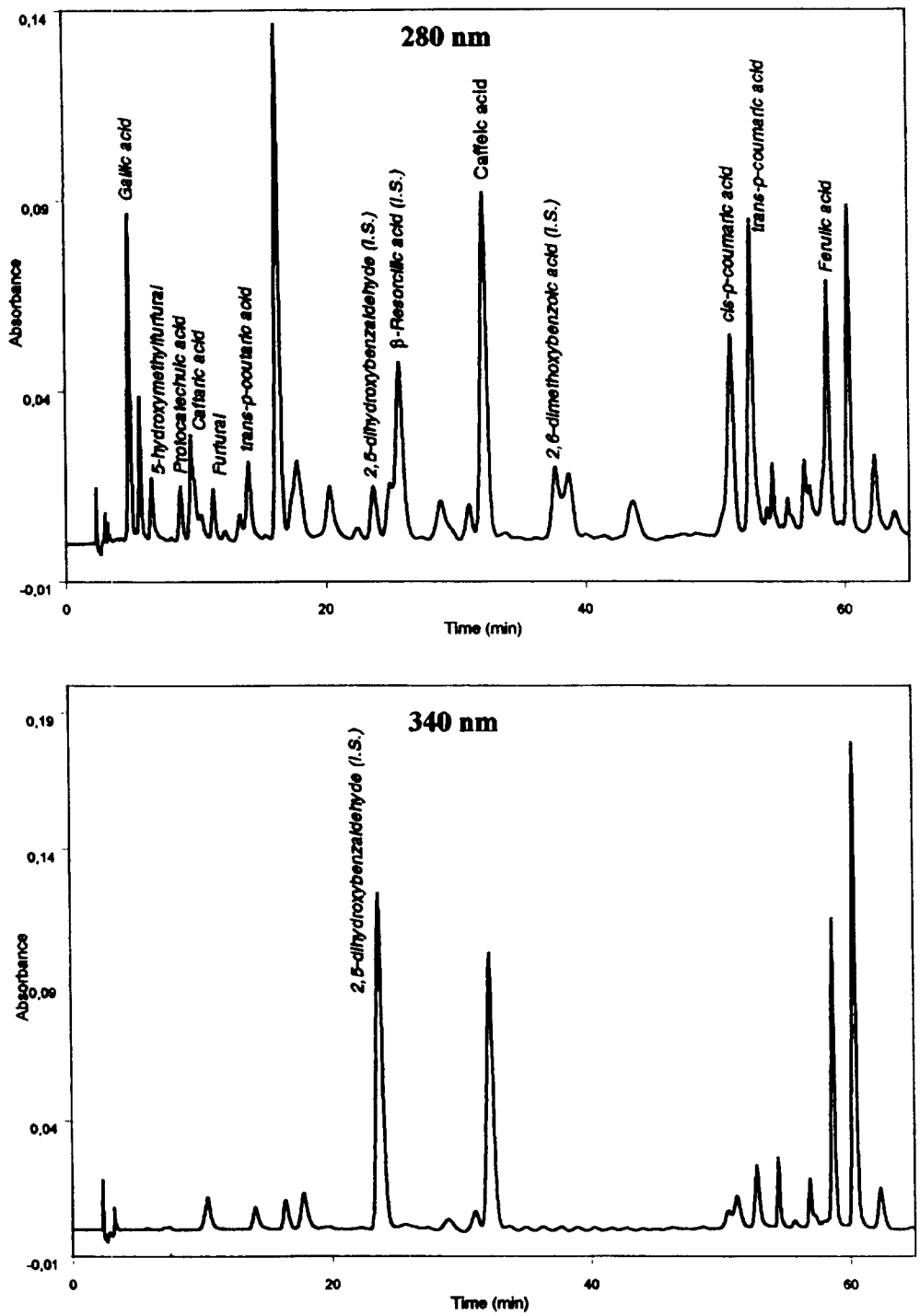

Fig. 4. Chromatograms obtained at 280 and 340 nm from a sample of wine using 2,5-dihydroxybenzaldehyde, β-resorcylic acid and 2,6-dimethoxybenzoic acid as internal standards.

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