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High-performance liquid chromatographic analysis of polyphenolic compounds predominating in sherry musts[☆]

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ABSTRACT

A chromatographic procedure has been developed for the analysis of polyphenolic compounds in sherry musts. The procedure allows for the use of internal standards to control the different steps of the analysis. A C_{18} reversed-phase column of 25 cm length and 4 mm diameter charged with 5 μm particle size filling is used in connection with a gradient in stages consisting of a 2% acetic acid solution in methanol. The choice of the gradient has been previously worked out as applied to a mixture of 22 polyphenolic compounds. In order to ensure the stability of the must samples they are mixed with 10% dimethylformamide and 2% ascorbic acid. The internal standard is then added to the samples, which are subsequently submitted to a continuous rotary extraction process for 3 h using diethyl ether.

INTRODUCTION

The importance of polyphenolic compounds in enology is well established [1]. Particularly in connection with sherry wines, such compounds are related to undesirable phenomena, such as the typical "browning", that occurs as a result of the ageing processes of these wines [2].

A high proportion of such compounds arise directly from the grapes so that the process of wine making, greatly influences the final polyphenolic content [3]. As a result it is of considerable interest to study these compounds in must samples taken from vintages as well as to follow their evolution during the wine-making process.

For reasons directly related to the complexity of the samples, in the analysis of these compounds it is mandatory to carry out a preliminary extraction of polyphenolic compounds, which are then determined by HPLC [4,5]. This stage of sample preparation is based mainly on liquid–liquid extraction techniques [6,7], solid-phase extraction [8–10], gel permeation chromatography (GPC) [11,12] or even chromatography on an open column [13]. In all cases considerable sample manipulation occurs, leading to the occurrence of a number of errors. Therefore, it is of particular interest to be able to control the quality of analyses, which can be achieved provided one or more internal standards can be added to the sample at the start of the analytical process. On the other hand, the great number of polyphenolic species that are dealt with finally by HPLC imposes the need to select a suitable separation process allowing for the inclusion of the internal standards that will appear in the final chromatogram.

The present work is concerned with the possibility of using internal standards with a view to controlling the overall analytical process of poly-

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phenols in must samples, carrying out the final quantification by HPLC. For this purpose a previously selected gradient is applied as worked out in connection with 22 polyphenolic species, allowing for the inclusion of internal standards in the must samples to be analysed.

EXPERIMENTAL

Reagents and standards

The standard compounds used were obtained from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany) and Eastman Kodak (Rochester, NY, USA). Methanol of HPLC-gradient grade and all other chemicals used (analytical-reagent grade) were obtained from Merck (Darmstadt, Germany). Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Preparation of the must samples

The samples of the investigated musts were obtained directly from the winery of "Bodegas Osborne & Cia" in different stages of processing: pressing, cleaning by sedimentation and fermentation.

Extraction of the samples was carried out under the cautionary conditions described elsewhere [14] in order to guarantee their stability throughout the different stages of the analysis: manual filtration with cotton cloth followed by the direct addition of 10% dimethylformamide (DMF) and 2% ascorbic acid and immediate freezing until the moment of analysis.

Extraction of polyphenolic compounds from the must

The extraction process was carried out by means of a continuous extraction rotary device, as designed by the authors [15], by adhering to the following procedure. A 1-ml volume of a 150 mg/l solution of 2,5-dihydroxybenzaldehyde (Fluka) was added to 100 ml of must followed by the addition of 1000 ml of water, thereafter saturating the obtained solution with NaCl. The solution was extracted with 80 ml of diethyl ether using a Mascré extractor for 3 h at a rate of 0.8 turns/min. The organic solvent was dried for 1 h with anhydrous sodium sulphate followed by evaporation in a rotavapor to attain a final volume of 5 ml.

TABLE I

GRADIENT ELUTION PROGRAMME

Time (min)	0	25	45
Solvent B (%)	0	15	50

Equipment

A Waters (Millipore, Milford, MA, USA) liquid chromatograph with Model 510 and M45 pumps, a Model M680 gradient programmer, a Model U6K injector and a Model M991 diode-array detector were used. Separation was carried out using a LiChrospher C_{18} steel cartridge, 250×4 mm I.D. and 5 μm particle size filling (Merck).

Chromatographic conditions

The chromatographic conditions adopted were as follows: flow-rate, 1 ml/min; detection, UV absorption at 210–390 nm; volume injected, 20 μl; and mobile phase, methanol–acetic acid–water (10:2:88, v/v) as solvent A and methanol–acetic acid–water (90:2:8, v/v) as solvent B with a gradient programme in two steps (Table I).

RESULTS AND DISCUSSION

As a rule, one of the most important stages of the analytical procedure used by the authors in the past for the chromatographic separation of polyphenolic species by HPLC was carried out using a 30-cm-long C_{18} column of irregular silica and 10 μm particle size [16]. Because of the great complexity of the sample, such a procedure precludes the use of internal standards that cannot be quantified reliably. Fig. 1a shows a chromatogram obtained from a must sample under the specified conditions, which clearly demonstrates the impossibility of including any internal standard.

In view of such difficulties a search was carried out for a reversed-phase column of more theoretical plates coupled with an elution gradient that would afford the inclusion of some internal standard. As a result five reversed-phase

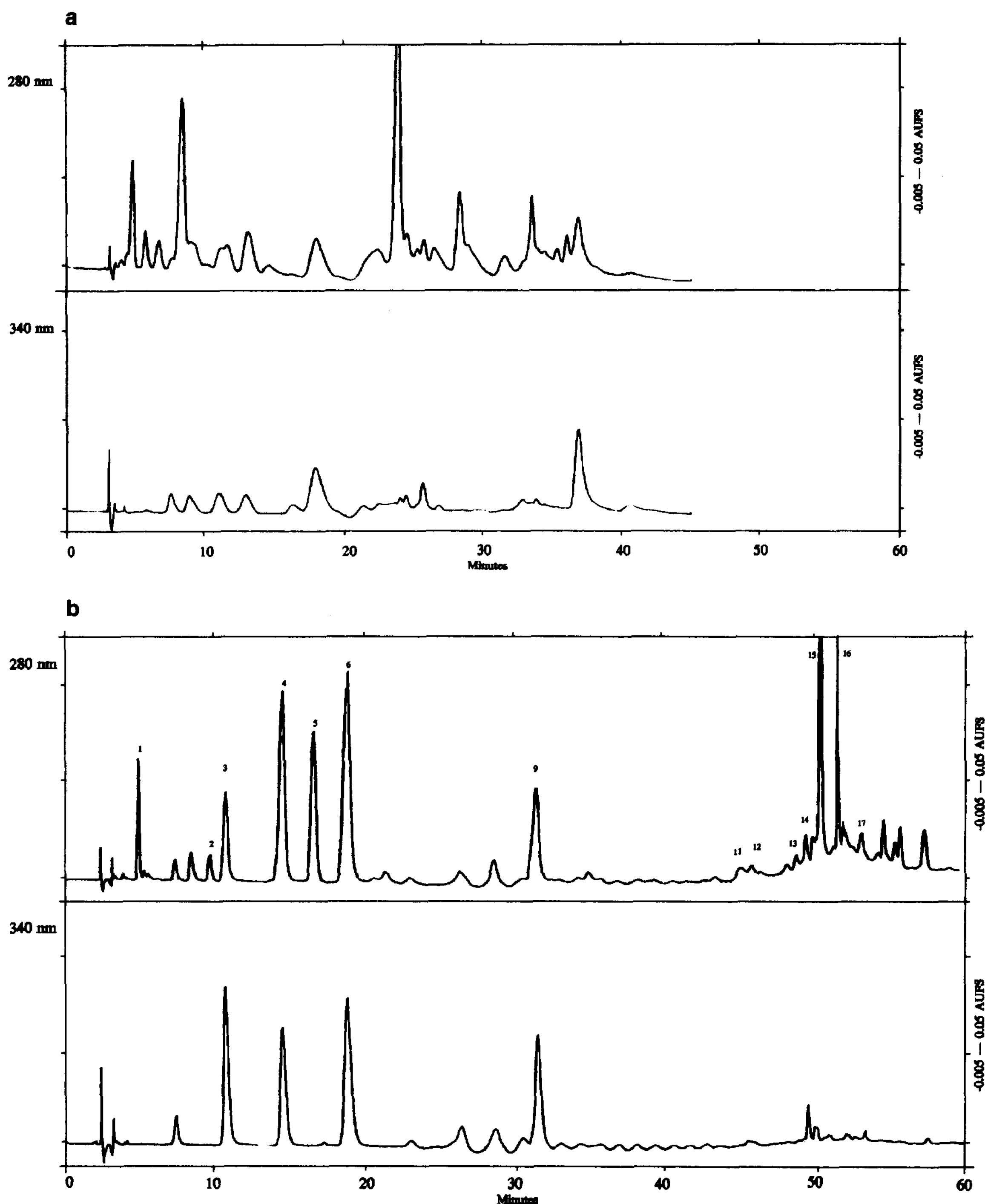

Fig. 1. Chromatograms obtained from the must sample (280 and 340 nm). The substances are identified in Fig. 2. (a) Chromatographic conditions: C_{18} column, 10 μm particle size, 30 cm length and the gradient described in ref. 16. (b) Chromatografic conditions: C_{18} steel cartridge, 5 μm particle size, 250 × 4 I.D. mm and the gradient described in the chromatographic procedure.

columns of different lengths and particle sizes were compared, determining the optimum elution gradient for each one [17]. Finally, a LiChrospher C_{18} steel cartridge, 250 × 4 mm I.D. with 5 μm particle size filling (Merck), was selected. Using this column in connection with the gradient as described before, the chromatogram reproduced in Fig. 1b was obtained for the same must sample.

The selected chromatographic conditions allow a better peak resolution. In addition, the void central zones, which can be easily appreciated in the chromatogram, hint at the possibility of including some polyphenolic substance (internal standard) that does not overlap with other compounds present in the must sample or at least able to be quantified reliably. Of course, such an internal polyphenolic standard must be absent from the analysed must samples.

Selection of internal standards

As a result of the preliminary investigations carried out by the authors, three compounds were chosen as potential internal standards: two phenolic acids (β-resorcilic acid; 2,6-dimethoxybenzoic acid) and one phenolic aldehyde (2,5-dihydroxybenzaldehyde). These compounds were added to a given sample of must and resulted in the chromatograms reproduced in Fig. 2, which allows the conclusion that two of the three tested compounds can be used as internal standards (2,6-dimethoxybenzoic acid, quantified at 280 nm, and 2,5-dihydroxybenzaldehyde, quantified at 340 nm).

Bearing in mind that the composition of the must samples in terms of polyphenolic species may vary widely, depending on the stage of the wine-making procedure, the chosen polyphenolic substances (internal standards) should be equally useful for any type of must sample arising during the wine-making procedures.

Application to different samples of must in the wine-making procedure

Taking into account the considerations described above, the selected standards were in-

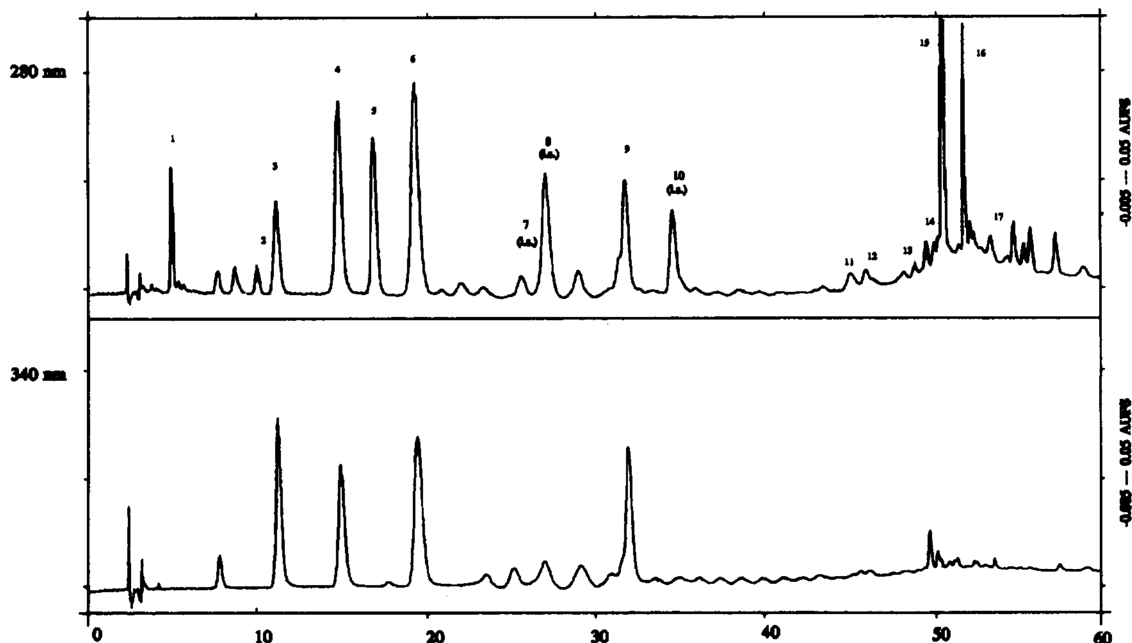

Fig. 2. Chromatograms (280 nm and 340 nm) obtained from must sample with addition of three internal standards. Peaks: 1 = gallic acid; 2 = protocatequialdehyde; 3 = *trans*-caffeoyltartaric acid; 4 = feruoyl tartrate; 5 = catechin + unknown; 6 = *p*-coumaroyl tartrate; 7 = 2,5-dihydroxybenzaldehyde (I.S.); 8 = β-resorcylic acid (I.S.); 9 = caffeic acid; 10 = 2,6-dimethoxybenzoic acid (I.S.); 11 = veratraldehyde; 12 = *p*-coumaric acid; 13 = 2,4-dimethoxybenzoic acid; 14 = ferulic acid; 15 = anisaldehyde; 16 = veratric acid; 17 = 3,4,5-trimethoxycinamic acid.

vestigated using a number of samples taken from a must deposit during different stages or processes of wine-making. The chromatograms obtained are reproduced in Fig. 3, and show different profiles depending on the wine-making process considered. The chromatograms of fermented musts (Fig. 3b) show a number of new peaks appearing in the region of the internal standard added (2,6-dimethoxybenzoic acid), which in some cases result in peak overlapping. Despite this, 2,5-dihydroxybenzaldehyde can be used as an internal standard to follow the wine-making process.

Once it was established that this compound can be used as an internal chromatographic standard, a study was carried out to determine its behaviour from the beginning, through sample preparation up to its final quantification by HPLC. With this aim a series of must samples were submitted to the extraction procedure and then added to a known concentration of the internal standard selected. The resulting chromatograms indicate that recovery of the standard was 98.9%, with a relative standard deviation of 1.98%. As a result the suitability of such a substance as a means of controlling the quality of the overall analytical procedures was confirmed.

CONCLUSIONS

Using a C_{18} steel cartridge (250 × 4 mm I.D.) and 5 μm particle size filling and the chromatographic conditions specified in the present paper, it is possible to use 2,5-dihydroxybenzaldehyde as an internal standard, quantified at 340 nm, for the analysis of phenolic compounds in the sherry

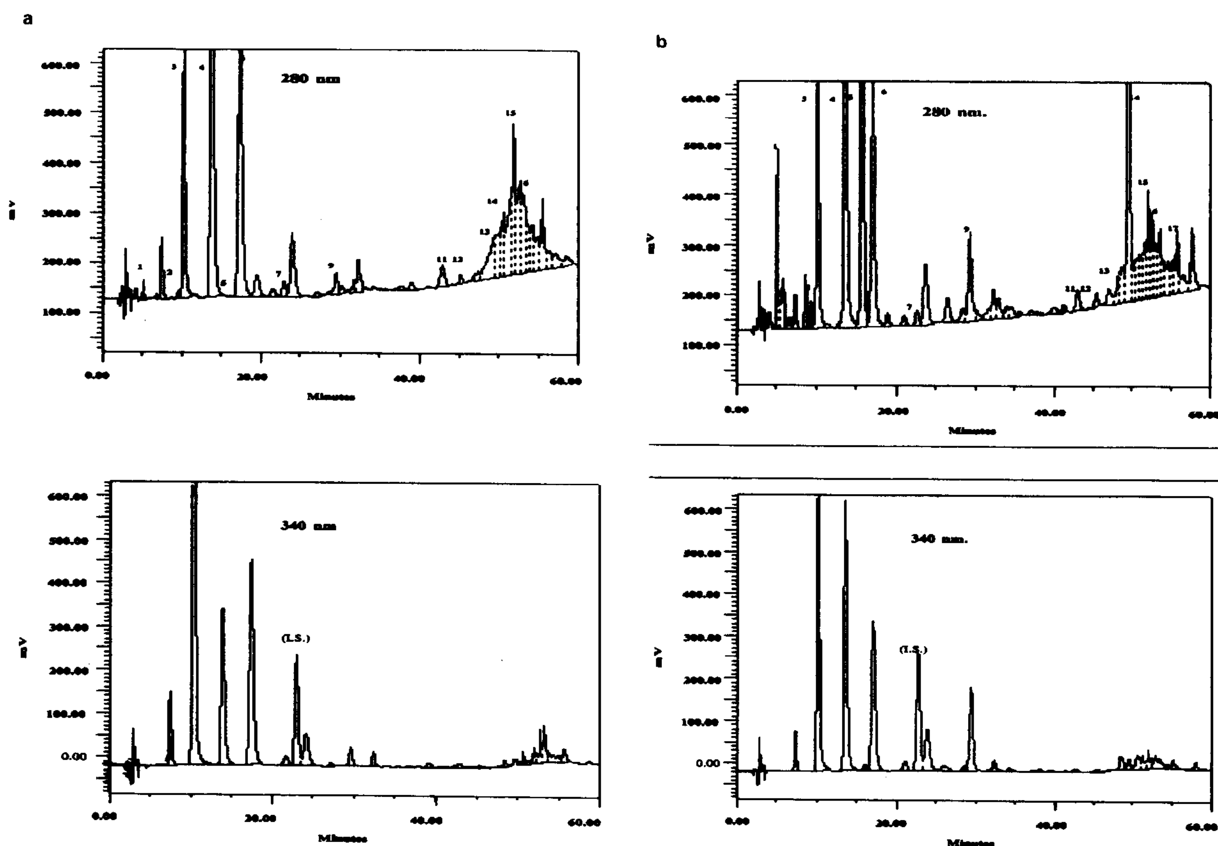

Fig. 3. Chromatograms (280 nm and 340 nm) obtained from different samples of must in the wine-making procedure, using 2,5-dihydroxybenzaldehyde as internal standard. (a) Must from the pressing stage. The substances are identified in Fig. 2. (b) Fermented must.

musts. In addition, this compound can be used to control the analytical steps preceding the HPLC analysis because the extent and relative standard deviation of its recovery are satisfactory. Such a possibility allows the efficiency of one of the stages of sample preparation to be checked, for instance the extraction step, which is a process highly related to sample manipulation.

In summary, we conclude that the proposed methodology can be used in connection with studies related to the control and evolution of the wine-making processes of sherry musts.

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