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Automation of sample preparation as a preliminary stage in the high-performance liquid chromatographic determination of polyphenolic compounds in sherry wines

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Abstract

The procedure for automating the method of sample preparation by solid-phase extraction prior to the HPLC analysis of polyphenolic compounds in sherry wine is described. This method consists of two stages, the first being sample preconcentration and cleaning using a C_{18} cartridge and the second fractionation using a SAX anion exchanger. The first direct application of the method, using a semi-flexible robot system, did not produce satisfactory results, thus requiring a series of modifications and optimization of the variables inherent in the device itself. The automated method as modified produced considerable improvements over the manual method with respect to the recovery and repeatability values obtained with the different polyphenolic species used in its optimization. Subsequent testing of the method with real samples of sherry wine proved its applicability for tracking the evolution of polyphenolic species in wine.

Keywords: Wine; Sample preparation; Sherry wine; Polyphenolic compounds

1. Introduction

In all foods of vegetable origin a wide variety of polyphenolic species are present, influencing strongly the organoleptic properties of the food and contributing in great measure to the processes of degradation [1]. In the case of "fino" sherry wines, their role is widely known and acknowledged [2].

Since samples of wine are highly complex, analytical techniques such as HPLC are necessary [3–5]. However, even when this technique is used in association with detection methods with a high discrimination power like UV diode-array detection, because of the great variety of species present and the wide variations in their levels, it is essential to devise a sample preparation stage which will ensure reliable identification and quantification.

Techniques such as chromatography in open columns using polyamide [6] and gel permeation chromatography [7] have been applied to isolate polyphenolic compounds, although the method possibly most often used is liquid–liquid extraction with diethyl ether, either in stages [8] or in continuous rotation [9]. Other workers have used extraction with ethyl acetate, adjusting the pH to 7 and 2 to separate the species into two groups, but, even with this method, the chromatograms obtained are relatively complex [10–12]. An

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alternative to the above-mentioned techniques is solid-phase extraction (SPE), which offers several advantages, including better selectivity and a faster speed, and is easier to automate [13,14]. There are already numerous reports of the application of this technique to sample preparation prior to HPLC analysis of polyphenolic species in various foods [15–17], grapes [18], musts and wines [19–21].

Using SPE, we have developed a scheme for the fractionation of polyphenolic substances consisting of two stages: in the first, the sample is preconcentrated and cleaned using a C_{18} cartridge; in the second, it is fractionated into two groups, acidic species and neutral species, by means of a SAX anion exchanger [22]. It is predictable that the possible automation of the fractionation scheme would permit more reproducible results to be achieved, compared with those obtainable from the use of vacuum equipment.

This paper presents the work undertaken to adapt the fractionation scheme to permit its use with an automatic, semi-flexible robotic system, namely the BenchMate work station from Zymark.

2. Experimental

2.1. Reagents and standards

The internal standards used to develop the method were obtained from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Eastman Kodak (Rochester, NY, USA) and Extrasynthèse (Genay, France); they were dissolved in a matrix medium [15% (v/v) ethanol–3 g/l tartaric acid in water] at concentrations similar to those described in real samples of wine (Table 1). The polyphenolic compounds used as internal standards were 2,5-dihydroxybenzaldehyde and 2,6-dimethoxybenzoic acid, at concentrations of 200 and 300 mg/l. Methanol (HPLC-gradient grade) and all other reagents were obtained from Merck. The water used throughout was of HPLC quality, purified in a Milli-Q apparatus (Millipore, Bedford, MA, USA). Before being used, all the solutions prepared were filtered through 0.45-μm membranes (Millipore) and degasified in an ultrasonic bath.

Table 1
Composition of the mixed solution of standards

Compound	Concentration (mg/l)
Gallic acid	4.24
Protocatechuic acid	5.40
Protocatechualdehyde	2.88
Gentisic acid	33.40
p-Hydroxybenzoic acid	7.32
Catechin	17.26
2,5-Dihydroxybenzaldehyde	16.82
p-Hydroxybenzaldehyde	1.92
m-Hydroxybenzoic acid	5.80
Vanillic acid	2.42
Caffeic acid	2.44
2,6-Dimethoxybenzoic acid	6.64
Syringic acid	2.32
Vanillin	6.72
Epicatechin	18.1
Syringaldehyde	3.20
p-Coumaric acid	1.72
o-Vanillin	6.28
Ferulic acid	2.80
Sinapic acid	6.28
o-Coumaric acid	1.52
3,4,5-Trimethoxycinamic acid	2.12
3,5-Dimethoxybenzaldehyde	5.20

2.2. Wine samples

The samples of wine were obtained directly from the wine-producing company, Osborne (El Puerto de Santa Maria, Cádiz).

2.3. Solid-phase extraction

The adsorbents used were LiChrolut C_{18} and SAX from Merck, in both cases with 500 mg of filling in a bed volume of 2.8 ml.

2.4. Chromatographic equipment and conditions

All the controls were carried out by HPLC, injecting the fractions collected into a Waters chromatograph (Millipore) consisting of two Model M510 pumps, a Model 717 automatic

injector, a Model M996 photodiode-array detector, scanning between 230 and 390 nm, and a Millenium 2010 chromatographic control and data handling system. The separation was performed using a LiChrospher C_{18} steel cartridge column (25 cm × 4 mm I.D., particle size 5 μm) (Merck). The chromatographic conditions employed were those established previously [21], injecting into the chromatograph 40 μl in the case of the acidic polyphenol fractions and 60 μl for the neutral polyphenol fractions. The identification of the peaks in the real wine samples was effected by comparing their UV spectra with the spectral library made by the authors [23].

The semi-flexible robotic system used to automate the sample preparation process was a BenchMate workstation from Zymark (Hopkinton, MA, USA).

3. Results and discussion

In order to automate a previously developed SPE procedure, the chemistry of the method is initially left unchanged; it is only a question of optimizing the parameters to be used in the automatic method, specific to the actual equipment to be used. i.e., the flow-rates for the conditioning, the loading, the washing, the elution, etc.

On this basis, when our method previously designed for a manual apparatus was applied directly to the automatic system, accepting the flow-rates offered by the equipment used owing to a lack of alternatives, acceptable results were not obtained, as the recoveries for the species studied were all below 15%. It was therefore concluded that the two stages should be studied separately to identify the causes of such low recoveries, and subsequently to modify each stage to obtain a better recovery.

3.1. Optimization of the sample preconcentration and cleaning stage

The first experiment performed was to determine the level of recovery from the stage of cleaning and preconcentration using a C_{18} cartridge, under the conditions of the manual method; this produced a generally low level of recovery, especially of the more polar species.

With a view to increasing the recovery, the effect was tested of adding an ion pair-forming reagent to the sample and to the phase used to condition the extraction cartridge. A series of experiments were carried out using tetrabutylammonium bromide, modifying the working pH level and either saturated or unsaturated with NaCl. It was clearly seen that the addition of this modifier, in a medium of pH 6.5 and saturated with NaCl, caused a significant increase in the recovery of the species studied, and more notably of the more polar species, such as gallic acid and protocatechuic acid; among these species, the recovery was still low but had been increased to two and three times the former levels.

Next to be studied were the flow-rates and phase volumes to be used in the different stages, so that the maximum recovery could be achieved in the desired final volumes.

The best conditions established for sample cleaning and preconcentration stage are given in Table 2.

3.2. Optimization of the fractionation stage

As for the first optimization, the first experiment was performed applying the same conditions as in the manual method and accepting the flow-rates from the first stage. Good results were obtained for the acidic polyphenol fraction but for the neutral fraction the yields were low.

It was logical to assume that the low recovery of the neutral species was due to some part of the fraction still remaining in the SPE cartridge or in the equipment conduits; therefore, one possibility for a better recovery would be to collect the liquid from the wash which was performed after the loading of the column. However, owing to the rigid programming of the equipment, this possible procedure is laborious to perform, since the software instructs the equipment to send the wash liquids from the cartridge direct to waste. It was therefore necessary to divide the procedure into two parts which

Table 2
Operating conditions for cleaning and preconcentration stage

Parameter	Conditions	Notes
Adsorbent	C_{18} (500 mg) Solvated with 10 ml of methanol Conditioned with 3 ml of a solution of 0.025 *M* tetrabutylammonium bromide saturated with NaCl	
Sample	Sample volume: 5 ml Added to the sample is a volume of 0.2 ml of a solution of the internal standards Added next to the sample is a volume of 5 ml of a solution containing 25 g of 0.025 *M* tetrabutylammonium bromide saturated with NaCl The contents of the tube are shaken for 30 s A volume of 9.8 ml of the resulting sample solution is loaded into the cartridge	
Washing solvent	The cartridge is washed with 0.6 ml of phosphate buffer (pH = 6.5, $I = 0.05$ *M*)	
Cartridge drying	With He for 150 s	
Elution	(1) 1.2 ml of methanol (2) 2 ml of phosphate buffer (pH 6.5, $I = 0.05$ *M*)	This eluate corresponds to the polyphenol compounds
Flow-rates	Conditioning: 0.25 ml/s Loading: 0.01 ml/s Washing: 0.10 ml/s Elution: 0.10 ml/s Air: 0.25 ml/s Air factor: 0.8	

were later linked, in such a way that the loading of the column and the washing would be performed for all the samples; the retained acids would be left in the adsorbent and subsequently eluted for all the samples together. The best conditions established for the fractionation stage are given in Table 3.

3.3. Repeatability study

When the two stages of the process had been adapted, a study was undertaken to determine the repeatability of each stage separately and then for the combined scheme. In all cases, the study was repeated six times using synthetic solutions of standards, with concentrations and media as close as possible to those which would be found in real samples of wine and in the different stages of the process.

During all the experiments the gravimetric confirmation system of the workstation was activated in order to control precisely and measure the errors which might be made in the manipulations of liquids performed. The results are given in Table 4. As can be seen, in general terms the method behaved reasonably well, although poor results were obtained for certain species, such as gallic acid and protocatechualdehyde which are recovered in very low proportions and with a low repeatability.

Table 3
Operating conditions for fractionation stage

Parameter	Conditions	Notes
Adsorbent	SAX (500 mg) equilibrated with 5 ml of water	
SAX procedure—first part		
Sample	The eluate obtained in the previous stage	The eluate in which the neutral polyphenols should be present is collected in this step
Washing solvent	1 ml of water	The column is washed with 1 ml of water which is collected in the same tube
SAX procedure—second part		
Cartridge drying	He for 300 s	
Elution	(1) 1 ml of 1 *M* HCl	This eluate corresponds to the acidic polyphenol fraction
	(2) 1 ml of water	
Flow-rates	Conditioning: 0.25 ml/s Loading: 0.01 ml/s Washing: 0.10 ml/s Elution: 0.10 ml/s Air: 0.25 ml/s Air factor: 0.8	

3.4. Application to samples of different types of sherry wine

For this study, samples of "fino", "amontillado" and "oloroso" wine, representing wines with increasing degrees of oxidation, were taken. Aliquots of 5 ml were taken and submitted to the sample preparation process. The fractions collected were injected into the HPLC system. When the chromatograms had been produced, the purity of the peaks was analysed and then they were identified by comparison with the library of UV absorption spectra; Figs. 1–3 show the results.

The most significant qualitative differences can be observed in the fractions corresponding to the neutral species; here, in the case of "amontillado" and "oloroso" wines, it is seen that two peaks appear at the beginning of the chromatogram. These were identified as 5-hydroxymethylfurfural and furfural, respectively, species which are produced by the dehydration of the sugars. Also in these types of wine, the appearance of peaks corresponding to syringaldehyde and vanillin, and also an increase in the heights of the peaks for *p*-hydroxybenzaldehyde, epicatechingalate and epigalocatechingalate, can be noted; these are species which are found more abundantly in the wood of the casks; one would logically expect this result, since these types of wine are aged for longer in wood and their higher alcohol content encourages the extraction of these compounds from the wood. One other important observation recorded in the chromatograms for "amontillado" and "oloroso" wines is the appearance of a very intense peak at 48 min, which succeeds in saturating the detector and the SPE cartridge as it appears in both fractions. This peak does not correspond to any known spectrum.

From the quantitative point of view, the chromatograms were reprocessed by the integration method which incorporated calibration against the internal standards 2,6-dimethoxybenzoic acid

Table 4
Repeatability study of automated sample preparation with the semi-flexible robotic system

Compound	Cleaning and preconcentration stage		Fractionation stage		Overall scheme	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Gallic acid	11.20	5.32	51.60	6.66	4.47	17.64
Protocatechuic acid	37.27	4.39	104.08	3.65	41.11	8.59
Protocatechualdehyde	38.62	3.74	10.78	10.58	4.90	19.70
Gentisic acid	102.37	1.99	98.74	4.55	104.86	3.19
p-Hydroxybenzoic acid	87.45	2.85	109.46	4.35	96.49	5.70
Catechin	82.73	16.85	69.25	18.72	58.24	22.25
2,5-Dihyidroxybenzaldehyde (I.S.)	100.47	3.90	84.62	2.38	66.16	11.97
p-Hydroxybenzaldehyde	104.16	3.18	82.99	1.16	88.16	3.23
m-Hydroxybenzoic acid	103.09	2.60	106.92	4.60	112.75	3.07
Vanillic acid	67.79	2.99	106.32	3.85	75.44	6.02
Caffeic acid	99.63	3.29	78.27	7.71	84.31	4.82
2,6-Dimethoxybenzoic acid (I.S.)	72.10	2.47	101.18	3.73	61.04	4.81
Syringic acid	49.06	2.82	106.12	3.95	53.80	5.72
Vanillin	96.56	2.40	80.34	1.64	80.83	4.50
Epicatechin	82.34	10.70	82.56	13.10	72.34	18.68
Syringaldehyde	87.13	8.05	77.95	0.90	68.91	4.89
p-Coumaric acid	103.16	2.62	98.06	5.67	107.04	3.50
o-Vanillin	100.29	4.63	81.02	1.08	84.55	3.00
Ferulic acid	98.54	2.06	94.37	5.95	93.59	3.31
Sinapic acid	89.54	4.01	44.83	9.23	35.60	7.35
o-Coumaric acid	105.35	2.34	48.24	7.20	67.85	4.52
3,4,5-Trimethoxycinnamic acid	98.45	2.37	94.03	6.54	82.88	4.34
3,5-Dimethoxybenzaldehyde	107.53	5.10	94.21	7.33	81.50	3.89

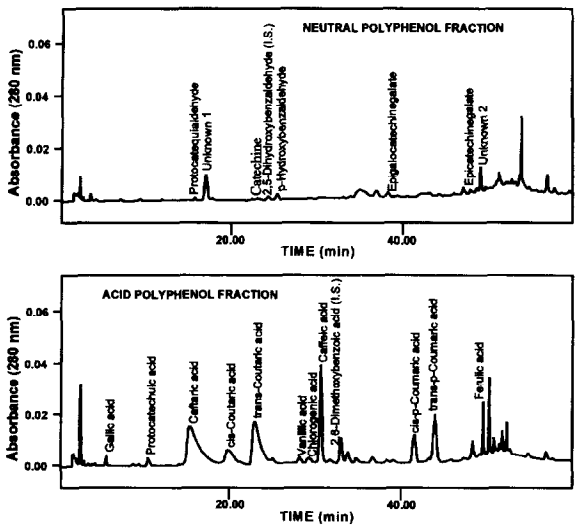

Fig. 1. Chromatograms (280 nm) of the fractions collected after application of the automatic SPE to samples of "fino" sherry wine.

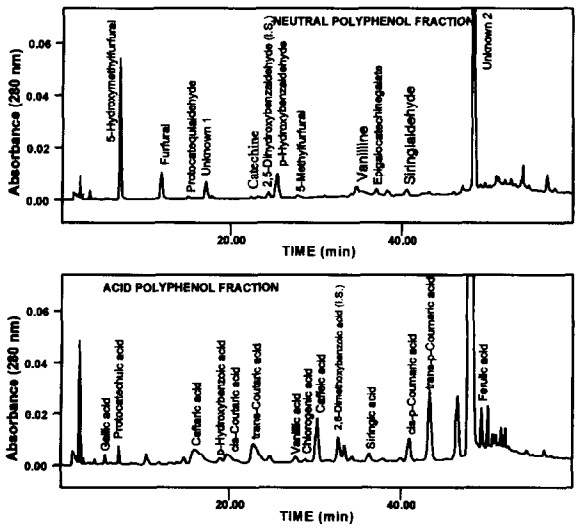

Fig. 2. Chromatograms (280 nm) of the fractions collected after application of the automatic SPE to samples of "amontillado" sherry wine.

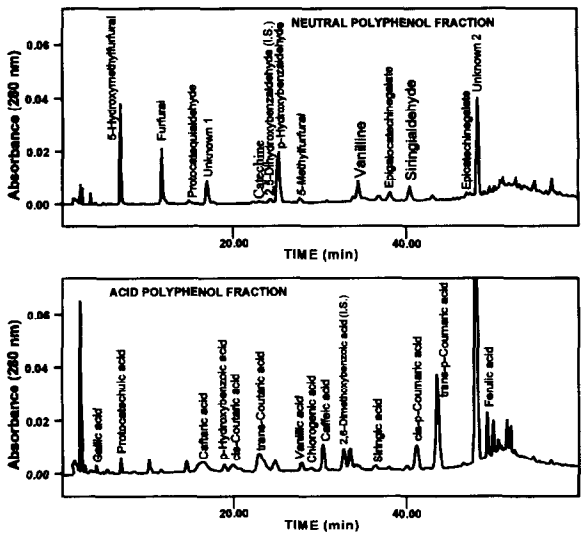

Fig. 3. Chromatograms (280 nm) of the fractions collected after application of the automatic SPE to samples of "oloroso" sherry wine.

and 2,5-dihydroxybenzaldehyde for the acidic and the neutral polyphenol fractions, respectively. The results obtained are given in Table 5.

From the analysis of these results, it can be concluded that the polyphenolic species in a combined state, the tartaric esters of caffeic and *p*-coumaric acids, tend to decrease as the degree of oxidation of the wine increases.

With respect to the free polyphenolic acids, protocatechuic, *p*-hydroxybenzoic, ferulic, vanillic and syringic acids are all observed to increase their levels in line with the degree of oxidation of the wine, considering "fino" as the least and "oloroso" as the most oxidized.

Other species detected such as *p*-hydroxybenzaldehyde, vanillin and syringaldehyde, are found in very low concentrations in "fino" wine, as would be expected for species present in the wood and extracted only gradually as the wine is aged longer in wood.

Table 5
Concentrations of the polyphenolic compounds identified in the different types of sherry wines

Compound	Concentration (mg/l)		
	"Fino"	"Amontillado"	"Oloroso"
Acidic polyphenol fraction			
Gallic acid	18.00	14.38	7.71
Protocatechuic acid	4.85	7.72	9.95
Caftaric acid[a]	19.22	4.41	5.94
p-Hydroxybenzoic acid	0.00	1.36	2.07
cis-p-Coutaric acid[b]	5.29	3.59	2.85
trans-p-Coutaric acid[b]	15.77	8.55	8.58
Vanillic acid	1.54	1.90	3.02
Chlorogenic acid[a]	3.50	1.52	1.32
Caffeic acid	12.79	6.92	4.47
Syringic acid	3.08	2.45	1.59
cis-p-Cumaric acid[b]	3.47	3.21	3.74
trans-p-Cumaric acid	4.93	7.60	10.96
Ferulic acid	4.05	4.91	5.19
Neutral polyphenol fraction			
Protocatechualdehyde	1.24	1.94	3.51
p-Hydroxybenzaldehyde	0.27	0.74	2.21
Catechin	16.46	4.50	3.74
Vanillin	0.20	1.31	1.72
Syringaldehyde	0.45	1.17	3.81

[a] Determined using calibration graph for caffeic acid.
[b] Determined using calibration graph for *trans-p*-coumaric acid.

4. Conclusions

The modified automated method produced considerable improvements over the manual method with respect to the recovery and repeatability values obtained with the different polyphenolic species used in its optimization. Subsequent testing of the method with real samples of sherry wine demonstrated its applicability for tracking the evolution of polyphenolic species in wine.

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