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Determination of free molecular phenolics and catechins in wine by solid phase extraction on polymeric cartridges and liquid chromatography with diode array detection☆

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Abstract

The use of HLB Oasis polymeric cartridges to extract phenolic acids and aldehydes from red wines has been assayed and compared with the combination of ODS and SAX cartridges. The recoveries on the polymeric cartridges resulted to be notably higher with good precisions. The best operation conditions (cartridge conditioning, sample volume, clean-up and elution) for these latter were selected by experiments carried out on a synthetic wine sample spiked with 14 compounds and on red wine samples. A matrix-matched calibration was advisable to reduce the influence of the matrix in the quantification of the analytes as it was verified from the application of standard addition calibrations on several wine samples. Determination of the analytes in the extracts was performed by reversed-phase HPLC using mobile-phase and flow gradients and detection at 250, 280 and 340 nm.

Keywords: Phenolic compounds; Solid phase extraction; Red wine

1. Introduction

Phenolic compounds influence the colour, astringency, bitterness, oxidation level and clarity of wines, and are also involved in the changes that take place during wine aging. Furthermore, catechins and proanthocyanidins contribute to the healthful properties of red wine. They act as antioxidants, scavenge free radicals that induce vascular relaxation and have anti-inflammatory, anti-carcinogenic and anti-mutagenic properties. They play important roles in the sensory qualities of the wines also [1–4].

The content of phenolic and volatile compounds in wine depends on grape composition, wine-making procedures, and enological techniques. In most cases, phenolic and volatile compounds extracted from wood are only a small fraction of wine phenolics and volatiles, and/or wine was aged in oak barrels or macerated with oak chips. The chemical composition of the wood barrels is influenced by many factors [5,6]. So, the concentrations and, even, the nature of the above-mentioned compounds are very variable between different types of wines [7,8].

Different techniques have been used for the determination of these compounds in wine samples; among others, thin layer chromatography and high-performance liquid chromatography have been the most used [8–13]. Recently, capillary electrophoresis has been applied to the separation of a wide variety of these compounds [14–18]. As regards the sample preparation, some authors use solid-phase extraction with ODS or strong anion-exchange (SAX) cartridges whereas others use liquid–liquid extractions with different organic solvents like ethyl acetate or diethyl ether, and some others inject the samples directly in HPLC without any preparation

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step [19–21]. A two-step extraction to fractionate the acidic and neutral compounds and a concentration of the sample, previous to the extraction, has also been carried out [22–27].

The aim of the present work is to assay the use of polymeric cartridges to extract the main low-molecular weight phenolic compounds, not bonded to other wine compounds, from wine samples. Its performance is compared against an usual procedure that involves the combination of ODS and SAX cartridges to obtain the acidic and neutral fractions of the analytes. Initially, the study is made on a synthetic wine, and then it is optimized and validated on red wines. The determination of the analytes in the extracts was performed by HPLC with diode array detection.

2. Experimental

2.1. Reagents and apparatus

HPLC quality acetonitrile, methanol and ethanol were supplied by Labscan (Dublin, Ireland). Acetic acid, hydrochloric acid, sodium chloride and ammonium hydroxide were purchased from Panreac (Barcelona, Spain). Gallic, *p*-coumaric, gentisic, ferulic, caffeic, syringic, vanillic and protocatechuic acids, protocatechualdehyde, *p*-hydroxybenzaldehyde, catechin and epicatechin standards were acquired from Sigma (St. Louis, MO, USA) and sinapic acid and *p*-vanillin were provided by Fluka (Buchs, Switzerland). Stock solutions of the analytes, and dilutions, were prepared in a 20:80 acetonitrile–water mixture. Water was previously purified in a Mili-Q system (Millipore, Bedford, MA, USA). The separation was carried out at 25 °C.

To carry out the extractions, 100 mg ODS and SAX cartridges were obtained from Isolute (Hengoed, Mid-Glamorgan, UK), 60 and 200 mg Oasis HLB cartridges were supplied by Waters (Milford, MA, USA). A SPE vacuum device from Isolute, which allowed to hand 20 samples simultaneously, was used. A rotary evaporator with water bath was supplied by Büchi (Plawil, Switzerland).

2.2. Preparation of a synthetic wine sample

The extraction procedures were studied by using mixtures of the phenolic acids and aldehydes dissolved in a matrix composed of a solution of tartaric acid (3.5 g/l) and ethanol, in proportions of 88 and 12%, respectively, in volume. The concentration of each analyte in the mixture was 25 mg/l.

Some experiments were also made with young and aged Spanish wines of different origin and with different analyte concentrations.

2.3. Solid phase extractions

2.3.1. Combination of ODS and SAX cartridges

A modification of the procedure proposed by Guillén et al., 1997 [28] has been applied. ODS cartridges were conditioned with 2 ml of methanol and 0.6 ml of a NaCl saturated solution at pH 2. Then, 1 ml of a 1:1 mixture of the synthetic sample and NaCl saturated solution at pH 2 was loaded onto the cartridge. The cartridge was cleaned-up with 0.2 ml of the NaCl solution and 0.2 ml of 0.01 M HCl and the analytes were eluted with 0.5 ml of methanol.

SAX cartridges were conditioned with 2 ml of water before loading them with the 0.5 ml eluate, previously mixed with 2.5 ml of a buffered phosphate solution of pH 6.5. The sample solution eluted through the cartridges was collected to analyze the neutral polyphenols. After the elution, the cartridges were rinsed with 0.2 ml of water and the acidic polyphenolic fraction was then eluted with 1 ml of 1 M HCl.

The final volume of each fraction was taken to 0.5 ml by using a rotary evaporator before HPLC analysis.

2.3.2. Oasis cartridges

The first procedure used to extract the analytes from the synthetic wine is the following. HLB Oasis cartridges (60 mg) were conditioned by elution of 1 ml of methanol and 1 ml of an ethanol–tartaric acid (3.5 g/l; 12:88) mixture. After that, a volume of 0.5 ml of synthetic wine containing the analytes was eluted by gravity. Then, the cartridges were rinsed with 0.5 ml of 1:1 methanol–water and eluted with two solvents to obtain two fractions. Firstly, the neutral compounds were eluted with 0.5 ml of acetonitrile–ammonia (2%; 90:10), finally, the acidic compounds were eluted with 0.5 ml of acetonitrile–acetic acid (5%; 90:10).

2.3.3. Experimental design using Oasis cartridges

Taguchi's L8 orthogonal arrays were used in order to elucidate the effect of the factors studied as well as the effect of double interactions among them. The statistical significance of the effects of the factors was determined by an analysis of the variance (ANOVA). The value of some variables that participate in the extraction of the analytes contained in the synthetic wine by using 60 mg Oasis was optimized. The Taguchi orthogonal array involves four two-level factors (Table 1).

The variables studied at two levels are the following: elution volume of each fraction, concentration of the acid and

Table 1
Taguchi orthogonal array

Trial	Column						
	A	**B**	**A × B**	**C**	**A × C**	**A × D**	**D**
1	1	1	1	1	1	1	1
2	1	1	1	2	2	2	2
3	1	2	2	1	1	2	2
4	1	2	2	2	2	1	1
5	2	1	2	1	2	1	2
6	2	1	2	2	1	2	1
7	2	2	1	1	2	2	1
8	2	2	1	2	1	1	2

Columns used to assign factors. **A**, NH_3 concentration (2–5%); **B**, AcOH concentration (2–5%); **C**, elution order (NH_3–AcOH); **D**, volume of each eluent (1–2 ml).

Table 2
Taguchi orthogonal array

Trial	Column						
	a	**b**	**c**	**d**	**e**	**f**	**g**
1	1	1	1	1	1	1	1
2	1	1	1	2	2	2	2
3	1	2	2	1	1	2	2
4	1	2	2	2	2	1	1
5	2	1	2	1	2	1	2
6	2	1	2	2	1	2	1
7	2	2	1	1	2	2	1
8	2	2	1	2	1	1	2

Columns used to assign factors: **a**, conditioning volume (2–5 ml); **b**, concentration of tartaric acid in the rinse solution (3.5–7 g/ml); **c**, wine sample volume (2–5 ml), **d**, volume of rinse solution (5–10 ml), **e**, percentage of organic solvent in the rinse solution (12–25%), **f**, solvent organic in the rinse solution (MeOH–EtOH), **g**, elution volume of each fraction (2–1 ml).

alkali and the order of the fraction elutions. So, the fractions were eluted with 1 or 2 ml of 10:90 acetonitrile–aqueous solutions where this latter solution was acetic acid or ammonia. The concentrations studied of acetic acid and ammonia were 2 and 5%.

Other experimental design was made to optimize the sample preparation procedure in order to analyze the compounds in red wine samples. In this case, 200 mg polymeric cartridges were used; these were conditioned with 3 ml of methanol and a volume of an alcohol–tartaric acid solution. The clean-up solvent was also different.

The variables and the levels considered were the volume of the 12:88 ethanol–tartaric acid (3.5 g/l) used to condition the cartridges (3 and 5 ml), the red wine sample volume (2 and 5 ml), the volume of the clean-up solution (5 and 10 ml), the nature of the alcohol in this rinse solution (ethanol or methanol) and its percentage (12 and 25%), the concentration of the tartaric acid aqueous solution (3.5 and 7 g/l) and the volume of the two eluents (1 and 2 ml for each one). In this case, the array involves seven two-level factors (Table 2).

2.4. HPLC determination

The chromatographic parameters of previous manuscripts have been modified to reduce the time analysis [18–20]. A Hewlett-Packard (Avondale, PA, USA) 1100 series liquid chromatograph coupled to a diode array detector was used in combination with a 200 × 4.6 mm i.d. Hypersil ODS column (particle size: 5 μm) from Phenomenex (Torrance, CA, USA). The best separation was achieved with a mobile phase and flow gradient: eluent **A** was acetic acid–water (2:98, v/v) and eluent **B** was acetic acid–acetonitrile–water (2:20:78, v/v); time 0 min, 100% **A**, flow 1 ml/min; time 55 min, 30% **A**, 70% **B**, flow 1 ml/min. The system was equilibrated by using the starting conditions for 10 min prior to injection of the next sample. UV–vis spectra (scanning from 190 to 400 nm) were recorded for all peaks. Quantification was made at 254, 280 and 340 nm. The injector was a Rheodyne with a sample loop of 10 μl.

3. Results and discussion

3.1. Chromatographic determination

Table 3 shows the data of the linear calibrations achieved by the injection of the standards solved in a mixture of acetonitrile–water (20:80). At this end, the peak areas were measured in the chromatograms obtained at the wavelengths of maximum absorption for each compound quantification wavelengths indicated in table. The variation of the retention times shown in table was that observed in the successive injection of 10 standards. The correlation coefficients (r^2) were always 0.99, at least for the stated concentration ranges.

The detection and quantification limits were estimated by successive dilution of a standard, considering a signal-to-noise ratio of 3 and 10, respectively. Detection limits varied from 0.05 to 4.3 mg/l. Fig. 1 shows the chromatogram of a

Table 3
Parameters of the linear regression and experimental detection and quantification limits for the studied compounds

		Retention time (min), n = 10	Quantification wavelength (nm)	Confirmation wavelength (nm)	Linearity (mg/l)	Intercept	Slope	Correlation coefficient (r^2)	LOD (mg/l)	LOC (mg/l)
1	Gallic acid	6.18 ± 0.05	280	254	5.0–60.0	29.655	26.943	0.9996	0.5	1.6
2	Protocatechuic acid	11.77 ± 0.07	254	280	1.0–7.5	0.156	33.355	1.0000	0.4	1.0
3	Protocatechualdehyde	17.30 ± 0.06	280	254, 340	0.3–2.5	0.913	29.969	0.9999	0.1	0.3
4	Gentisic acid	18.11 ± 0.09	340	340	1.0–15.0	5.122	12.620	0.9991	0.4	1.0
5	*p*-Hydroxybenzaldehyde	25.42 ± 0.09	280	254	0.1–5.0	3.090	73.755	0.9995	0.05	0.2
6	Catechin	26.58 ± 0.18	280	280	5.0–100.0	18.036	5.453	0.9964	1.5	4.3
7	Vanillic acid	27.52 ± 0.12	254	254	1.0–7.5	12.136	32.280	0.9991	0.4	1.0
8	Caffeic acid	30.64 ± 0.20	340	280	1.0–10.0	14.614	37.985	0.9992	0.3	1.0
9	Syringic acid	33.71 ± 0.18	280	254	1.0–7.5	9.160	27.416	0.9991	0.4	1.0
10	*p*-Vanillin	35.34 ± 0.15	280	254, 340	0.5–5.0	16.292	35.030	0.9988	0.2	0.5
11	Epicatechin	39.53 ± 0.27	280	280	5.0–60.0	12.36	3.723	0.9903	0.6	1.8
12	*p*-Coumaric acid	43.46 ± 0.27	280	280	1.0–7.5	37.34	40.425	0.9984	0.3	1.0
13	Ferulic acid	50.89 ± 0.27	340	340	0.1–5.0	11.85	33.143	0.9917	0.06	0.1
14	Sinapic acid	54.14 ± 0.30	340	340	0.5–5.0	13.04	19.752	0.9978	0.3	0.5

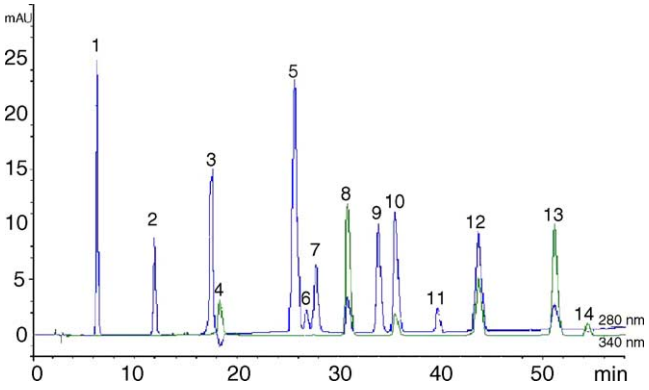

Fig. 1. Chromatogram obtained by using a Hypersil ODS column and the proposed mobile phase for a standard mixture (10 mg/l each one). See Table 3 for peak identification.

standard where the separation of the 14 compounds can be seen.

3.2. Comparison of solid phase extraction procedures

Table 4 lists the recoveries and precisions achieved in the extraction of the phenolic compounds from a synthetic wine sample by the two assayed sample preparation procedures. The use of both procedures supposes the elution of the analytes in two fractions, the first one containing the neutral compounds and the second one the acidic compounds.

As it can be seen in Table 4 the recoveries obtained by the ODS + SAX cartridges are very low for most of the compounds, lower than 10% by adding the recoveries of the two fractions, whereas the recoveries are notably higher using the Oasis cartridges. For these latter, most of the analytes coelute in both fractions and the recoveries are generally higher in the first fraction; gallic, protocatechuic and caffeic acids,

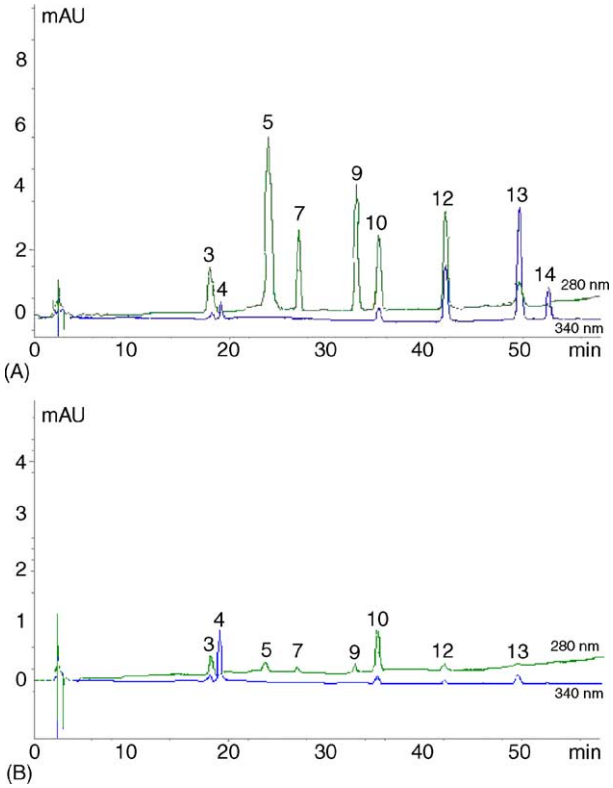

Fig. 2. Chromatograms of a synthetic wine sample obtained by solid phase extraction with 60 mg HLB Oasis cartridges. See Table 3 for peak identification. (A) Neutral fraction. (B) Acidic fraction.

catechin and epicatechin are not detected. Fig. 2 shows the chromatograms of the neutral and acidic fractions recorded in the injection of the Oasis extracts. As some analytes were distributed between the two fractions, the collection of the fractions on only a vial, and the injection of only an extract by sample, was considered as a better option.

Table 4
Recoveries and coefficients of variation (%) obtained in the extraction of the analytes from a synthetic sample by using different solid phase extraction procedures (n = 5)

	ODS + SAX procedure				OASIS procedure			
	Neutral fraction		Acidic fraction		Neutral fraction		Acidic fraction	
	Recovery	Precision	Recovery	Precision	Recovery	Precision	Recovery	Precision
Gallic acid	–	–	10	10	–	–	–	–
Protocatechuic acid	–	–	10	7.7	–	–	10	6.0
Protocatechualdehyde	–		9	5.4	25	5.7	10	11.5
Gentisic acid	–	–	3	3.2	27	4.5	60	2.5
p-Hydroxybenzaldehyde	8	10.8	8	8.4	34	4.6	5	13.3
Catechin	–	–	–	–	–	–	–	–
Vanillic acid	6	5.4	3	2.0	87	0.4	10	24.7
Caffeic acid	–	–	7	0.3	–	–	–	–
Syringic acid	1	7.9	2	1.9	82	0.2	8	68
p-Vanillin	5	6.8	7	2.4	52	0.8	22	8.1
Epicatechin	–	–	–	–	–	–	–	–
p-Coumaric acid	6	25.6	6	2.6	81	0.2	8	41
Ferulic acid	2	14.5	5	2.0	74	0.1	2	53
Sinapic acid	6	11.9	3	6.2	36	3.2	–	–

(–) Below detection limit.

Table 5
Recoveries (%) obtained in the analysis of a synthetic wine sample by using 60 mg Oasis HLB cartridges after carrying out an experimental design (n = 3)

Conditions		Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8
(A) NH_3 concentration (%)		2	2	2	2	5	5	5	5
(B) AcOH concentration (%)		2	2	5	5	2	2	5	5
(C) Elution order	First eluent	NH_3	AcH	NH_3	AcH	NH_3	AcH	NH_3	AcH
	Second eluent	AcH	NH_3	AcH	NH_3	AcH	NH_3	AcH	NH_3
(D)Volume of each eluent (ml)		1	2	2	1	2	1	1	2

Compounds	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8
Gallic acid	13	–	61	93	–	–	–	–
Protocatechuic acid	65	35	24	85	10	12	–	–
Protocatechualdehyde	75	72	52	88	15	25	–	–
Gentisic acid	94	47	90	108	66	43	52	27
p-Hydroxybenzaldehyde	65	68	84	95	46	24	–	–
Catechin	54	–	63	88	–	–	–	
Vanillic acid	101	94	99	104	108	104	103	99
Caffeic acid	79	–	91	101	4	–	–	–
Syringic acid	99	89	101	103	102	98	99	94
p-Vanillin	98	88	100	101	98	92	–	–
Epicatechin	55	–	67	97	–	–	–	–
p-Coumaric acid	101	91	101	99	102	99	98	95
Ferulic acid	99	79	100	101	87	76	78	74
Sinapic acid	80	15	86	104	–	–	–	

(–) Below detection limit.

3.3. Optimization of the extraction with polymeric cartridges

Some variables were studied to improve the extraction of the target-compounds on the 60 mg Oasis cartridges: the elution volume, which was increased in relation to the previous assays, the percentages of acetic acid and ammonia, and the elution order of the fractions. The two eluates were now joined. Table 5 describes the characteristics of the factorial design and shows the recoveries of the compounds obtained on the synthetic sample for each experiment made.

As it can be verified in Experiment 1 from table, the increase of the elution volumes enhanced the recoveries of almost all the analytes in comparison with the elutions with 0.5 ml. In the new conditions, the worst recoveries were obtained for gallic acid, catechin and epicatechin.

Experiments 3 and 4 provided the higher recoveries. They had in common the use of 5% HAcO and 2% NH_3 and differed in the elution order. The NH_3 concentration and its interaction with HAcO were the most important effects in the performance extraction. They explained the 50 and 29%, respectively, of the data variability. The best results were achieved in Experiment 4, which corresponds to an elution with 1 ml of 10:90 acetonitrile–acetic acid (5%) followed by 1 ml of acetonitrile–ammonia (2%; 10:90). The recoveries of the compounds are notably higher in these conditions. Fig. 3 shows a chromatogram after combining the fractions.

3.4. Determination of phenolics in wine

A red young wine was analyzed applying the extraction conditions selected after the above-mentioned study. The gallic and protocatechuic acids were completely loosed during the cartridge loading while other analytes were partially retained. This fact was observed by injecting the wine sample after eluting it through the cartridge and it was corroborated by injecting directly the wine sample in HPLC. Moreover, there were partial co-elutions of some analytes with co-extracted compounds. The identity of each compound was confirmed by comparing the retention times and ultraviolet spectra of the peaks in wine with those previously obtained by injection of standards.

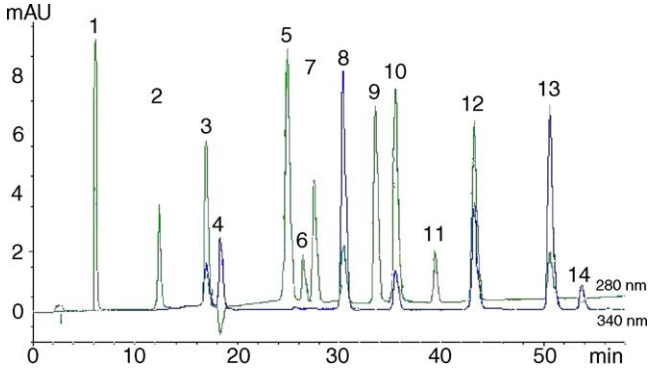

Fig. 3. Chromatogram of a synthetic wine sample obtained after carrying out the experimental design and combining the two fractions. See Table 3 for peak identification.

For the above reasons, Oasis cartridges of higher capacity, 200 mg, were chosen and a new experimental design was devised using as variables the conditioning volume, the sample volume, the percentage and nature of the organic solvent of the rinse solution, and the elution volume of each fraction.

As the eluent volumes were increased, a concentration step was included in the analytical procedure to determine lower

Table 6
Concentrations (mg/l) of the phenolic compounds in a young red wine sample obtained by using 200 mg Oasis HLB cartridges after carrying out an experimental design (n = 3)

Conditions	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8
(a) Conditioning volume (ml)	3	3	3	3	5	5	5	5
(b) Concentration of tartaric acid in the rinse solution (g/l)	3.5	3.5	7	7	3.5	3.5	7	7
(c)Wine sample volume (ml)	2	2	5	5	5	5	2	2
(d) Volume of rinse solution (ml)	5	10	5	10	5	10	5	10
(e) Percentage of organic solvent in the rinse solution (%)	12	25	12	25	25	12	25	12
(f)Solvent organic in the rinse solution	MeOH	EtOH	EtOH	MeOH	MeOH	EtOH	EtOH	MeOH
(g)Elution volume of each fraction (ml)	2	1	1	2	1	2	2	1

Compound	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8
Gallic acid	16.4	–	3.6	8.1	3.2	6.0	1.0	2.3
Protocatechuic acid	0.82	<LOC	–	0.69	<LOC	0.86	0.87	<LOC
Protocatechualdehyde	0.53	–	0.10	0.25	0.68	0.26	0.26	<LOC
Gentisic acid	–	–	–	–	–	–	–	–
p-Hydroxybenzaldehyde	–	–	–	–	–	–	–	–
Catechin	1.18	–	<LOC	0.57	–	<LOC	<LOC	<LOC
Vanillic acid	1.04	0.09	<LOC	<LOC	<LOC	0.50	0.72	<LOC
Caffeic acid	2.26	<LOC	–	1.96	–	1.81	1.72	–
Syringic acid	1.79	<LOC	–	1.57	–	1.52	1.61	0.11
p-Vanillin	1.89	–	–	1.06	–	0.75	1.19	–
Epicatechin	0.42	–	–	<LOC	–	–	<LOC	–
p-Coumaric acid	1.95	–	–	2.21	–	1.60	1.45	–
Ferulic acid	–	–	–	–	–	–	–	–
Sinapic acid	–	–	–	–	–	–	–	–

(–) Below detection limit. <LOC: below quantification level.

concentrations of the minor compounds. At this end, a rotary evaporator was used at a temperature of 36 °C and a pressure of 25 mbar.

Table 6 shows the concentrations obtained. The higher concentrations with simple relatively chromatograms were achieved after conditioning the cartridges with 3 ml of methanol and 3 ml of the ethanol–tartaric acid (3.5 g/l; 12:88) mixture, loading a sample volume of 2 ml, rinsing the cartridges with 5 ml of a methanol–tartaric acid (3.5 g/l; 12:88) mixture and eluting the analytes with 1 ml of acetonitrile–acetic acid (5%; 10:90) and 1 ml of acetonitrile–ammonia (2%; 10:90); these data can be seen in column of Experiment 1. The peak purity was checked for all the analytes in the chromatograms so that the concentrations stated in Table 6 were not high as consequence of the co-elution of interferences, although these latter affected to the integration of the chromatographic peaks. It is deduced from the results that the elution of the cartridges with 2 ml of each solution was necessary to increase the concentrations calculated. In fact, the elution volume explained a 73% of the variability, the contribution of the others factors was slight. Fig. 4 shows the chromatogram of the extract after the best experiment.

The array of extraction procedures has also been applied to other types of wine: white, rose and aged red wine. As it can be seen, the best results for the three different wines were achieved in Experiment 1 (see Table 7). This is the most appropriate procedure to extract the studied phenols in wines of different composition. The optimum experimental conditions are different in relation to those selected after the

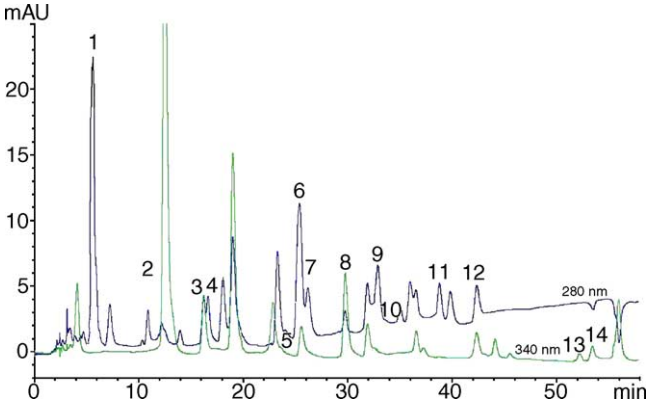

Fig. 4. Chromatogram of a phenolic compound extract from a young red wine obtained by the sample procedure proposed. See Table 3 for peak identification.

experiments made with the synthetic wine; these facts can be attributed to the different matrix of the samples.

3.5. Sample preparation procedure proposed

Briefly, the extraction and clean-up method that involves the use of 200 mg polymeric cartridges is the following. The cartridges were conditioned with 3 ml of methanol and 3 ml of an ethanol–tartaric acid (3.5 g/l; 12:88) mixture, then a wine sample of volume 2 ml was loaded and eluted by a suction system. The cartridges were rinsed with 5 ml of a methanol–tartaric acid (3.5 g/l; 12:88) solution and then the

Table 7
Concentrations (mg/l) of the phenolic compounds in different wines samples obtained by using 200 mg Oasis HLB cartridges after carrying out an experimental design ($n = 3$)

Compound	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8
White wine								
Gallic acid	0.57	0.06	0.25	0.18	0.28	0.37	0.44	0.42
Protocatechuic acid	0.45	0.09	0.10	0.35	0.17	0.41	0.41	0.20
Protocatechualdehyde	1.44	0.04	0.49	0.24	0.49	0.48	0.41	0.73
Gentisic acid	4.32	0.20	1.52	0.96	1.54	1.73	1.68	2.25
p-Hydroxybenzaldehyde	0.17	–	0.02	0.06	0.01	0.07	0.05	0.04
Catechin	11.23	–	0.44	2.38	0.42	2.39	2.34	3.79
Vanillic acid	0.19	0.05	0.04	0.23	0.07	0.27	0.27	0.07
Caffeic acid	3.52	0.47	0.36	3.61	0.90	3.59	3.66	0.85
Syringic acid	0.32	–	0.02	0.13	0.04	0.10	0.13	0.05
p-Vanillin	0.17	–	0.03	0.06	0.02	0.06	–	0.06
Epicatechin	16.95	–	1.19	8.91	1.85	11.06	6.24	0.84
p-Coumaric acid	0.82	–	0.03	0.66	0.08	0.67	0.64	0.09
Ferulic acid	0.52	–	–	0.43	0.06	0.44	0.47	0.06
Sinapic acid	–	–	–	0.144	–	0.14	–	–
Rose wine								
Gallic acid	4.96	0.10	1.48	1.76	0.89	2.50	1.78	3.12
Protocatechuic acid	2.09	0.47	0.65	1.31	0.45	–	–	0.75
Protocatechualdehyde	1.00	0.17	0.31	0.33	0.21	0.48	0.29	0.76
Gentisic acid	3.18	0.62	1.04	1.29	0.69	1.73	1.41	2.21
p-Hydroxybenzaldehyde	0.57	0.05	0.03	0.51	0.27	0.54	0.48	0.12
Catechin	12.83	0.21	0.59	9.21	0.49	9.41	8.86	3.54
Vanillic acid	0.65	0.17	0.25	0.64	0.17	0.69	0.64	0.25
Caffeic acid	1.07	0.30	0.40	1.12	0.25	1.07	1.09	0.40
Syringic acid	0.76	0.16	0.23	1.19	0.17	0.92	0.78	0.28
p-Vanillin	0.16	–	–	0.07	–	0.45	–	–
Epicatechin	18.6	1.38	3.90	7.83	2.65	14.46	8.14	3.01
p-Coumaric acid	0.78	0.12	0.18	0.77	0.10	0.74	0.75	0.17
Ferulic acid	0.17	–	0.04	0.13	0.24	0.13	0.17	0.55
Sinapic acid	0.14	–	0.07	0.18	0.42	0.21	0.21	0.17
Aged red wine								
Gallic acid	19.77	0.33	3.12	0.57	1.72	1.50	5.85	6.77
Protocatechuic acid	2.36	0.10	0.48	0.48	0.47	0.85	1.31	0.80
Protocatechualdehyde	1.13	0.05	0.15	0.01	0.10	0.12	0.35	0.55
Gentisic acid	3.81	0.21	0.50	0.34	0.38	0.46	1.32	1.70
p-Hydroxybenzaldehyde	1.73	0.05	0.11	0.57	0.01	0.73	1.05	0.13
Catechin	18.76	0.52	0.71	8.84	0.66	10.20	13.21	2.17
Vanillic acid	2.43	0.13	0.39	1.62	0.53	1.80	1.97	0.64
Caffeic acid	5.60	0.24	0.89	4.06	1.13	4.41	4.86	1.46
Syringic acid	3.09	0.11	0.43	1.93	0.63	2.29	2.22	0.32
p-Vanillin	0.41	–	–	0.18	0.02	0.29	0.27	0.05
Epicatechin	23.19	0.23	0.86	6.56	1.44	8.44	8.31	1.36
p-Coumaric acid	3.50	0.09	0.29	2.47	0.36	2.66	2.89	0.52
Ferulic acid	0.27	–	0.03	0.22	0.04	0.22	0.25	0.06
Sinapic acid	0.23	–	0.05	0.12	0.03	0.13	0.16	0.06

analytes were extracted with 1 ml of acetonitrile–acetic acid (5%; 10:90) followed by 1 ml of acetonitrile–ammonia (2%; 10:90). Finally, the two fractions were combined and evaporated to dryness in a rotary evaporator and the residue was dissolved in 0.5 ml of acetonitrile–water (20:80).

3.6. Application of the proposed method

Three different red wine samples (young, aged 1 year and aged 2 year wines) were subjected to the above-mentioned sample preparation. The analytes in the extracts were quantified by an external standard calibration with standards dissolved in acetonitrile–water, a matrix-standard calibration with extracts from synthetic wine samples spiked with increasing amounts of the analytes in the concentration range shown in Table 1 and a standard addition calibration. In this last case, microliter volumes of a standard solution were added to different extracts of the same sample, keeping virtually constant the extract volume. The correlation coefficients of the linear fittings were always 0.987, at least.

Table 8 shows the results obtained. In general, the concentrations estimated by the external standard conventional

Table 8
Concentrations (mg/l), and precisions (R.S.D.s, %), of phenolic compounds measured on three wine samples by external standard, matrix-standard and standard addition calibrations (n = 3)

	External standard		Matrix-standard		Standard addition	
	Concentration	Precision	Concentration	Precision	Concentration	Precision
Wine 1 (young)						
Gallic acid	65.03	2.0	65.67	2.0	64.65	1.1
Protocatechuic acid	8.24	13.7	7.95	9.1	6.57	1.8
Protocatechualdehyde	4.02	8.4	2.89	9.4	2.55	12.6
Gentisic acid	13.18	0.4	12.37	0.4	12.55	0.8
p-Hydroxybenzaldehyde	–	–	–	–	–	–
Catechin	31.21	14.1	31.26	16.1	30.25	8.3
Vanillic acid	3.22	14.6	3.20	13.9	1.99	16.4
Caffeic acid	12.33	3.8	11.51	4.1	10.87	3.8
Syringic acid	10.53	9.8	9.87	10.4	9.65	5.5
p-Vanillin	–	–	–	–	–	–
Epicatechin	21.36	31.5	20.92	22.4	19.83	10.5
p-Coumaric acid	7.56	18.7	6.94	14.0	6.32	5.4
Ferulic acid	2.19	14.2	1.2	21.2	1.43	10.6
Sinapic acid	6.16	9.5	4.6	11.5	4.26	6.5
Wine 2 (aged 1 year)						
Gallic acid	84.66	7.8	79.27	1.1	75.02	1.1
Protocatechuic acid	4.72	5.1	4.27	8.9	5.30	1.5
Protocatechualdehyde	1.85	11.1	1.87	10.0	2.49	3.2
Gentisic acid	5.82	6.5	5.14	3.7	5.06	0.9
p-Hydroxybenzaldehyde	–	–	–	–	–	–
Catechin	43.56	5.1	46.08	3.3	37.39	10.2
Vanillic acid	5.60	1.3	4.74	5.7	4.56	8.5
Caffeic acid	18.99	3.3	18.77	1.7	11.2	4.5
Syringic acid	8.03	0.6	7.21	4.1	6.52	4.3
p-Vanillin	5.7	10.5	3.4	10.0	2.4	11.2
Epicatechin	20.54	3.7	20.31	2.1	16.25	9.1
p-Coumaric acid	7.76	5.9	6.15	5.4	5.85	7.8
Ferulic acid	1.52	1.8	2.5	10.1	2.38	11.4
Sinapic acid	3.09	9.4	1.81	7.9	2.21	10.1
Wine 3 (aged 2 years)						
Gallic acid	59.42	4.6	55.93	4.7	52.52	2.1
Protocatechuic acid	1.90	21.3	1.71	22.4	1.87	3.4
Protocatechualdehyde	1.73	16.8	1.76	16.3	1.97	3.2
Gentisic acid	9.32	10.9	8.51	11.9	9.29	9.9
p-Hydroxybenzaldehyde	–	–	–	–	–	–
Catechin	60.31	10.2	64.52	10.9	55.45	10.2
Vanillic acid	4.15	5.3	3.44	6.3	3.97	6.5
Caffeic acid	12.49	2.2	11.67	2.3	5.12	7.8
Syringic acid	7.12	21.0	6.47	26.3	5.58	2.1
p-Vanillin	7.15	1.5	6.21	1.8	5.21	1.2
Epicatechin	11.32	10.1	10.53	12.5	9.01	9.1
p-Coumaric acid	3.36	12.1	1.61	25.1	2.23	10.4
Ferulic acid	1.42	19.6	0.73	42.8	0.98	15.2
Sinapic acid	1.15	3.6	–	–	–	–

(–) Below detection limit.

calibration were slightly higher than those achieved by the other calibration methods, which must be attributed to effects of the matrix. If the results obtained by the addition standard method are considered as correct, the concentrations obtained by the conventional calibration were about 2 mg/l higher for most compounds. The behavior was similar for the three types of wine as can be observed in Table 7. The matrix-standard calibration with extracts of spiked wine seemed to correct partially the quantitative errors. The testing of the peak purity allowed to verify that there were not co-elutions of interfering peaks at the retention times of the analytes.

The detection limits were established by dilutions of the extracts from the three types of wine where the analytes had been previously quantified, and considering a signal-to-noise ratio of 3. These limits ranged from about 0.5 to 4.0 mg/l, expressed as concentration in wine, except for *p*-hydroxybenzaldehyde, which was not found in these samples. The coefficients of variation of the analyses were comprised between 0.4 and 22%, after making the matrix-standard

and addition standard calibrations. Fig. 4 shows the chromatograms obtained for each type of wine; they were similar for the three wines analyzed.

Similar results about the calibration and detection limits were observed when wines of different colour (white and rose ones) were used to make these experiments.

3.7. Conclusions

The combination of ODS and SAX cartridges is not a valid alternative to retain the majority of the target analytes and obtain acceptable detection limits on wine samples. The use of 200 mg polymeric cartridges is a better option on account of the lower retention observed on 60 mg cartridges. The separation of the acidic and neutral compounds in two fractions is very poor by the assayed extraction procedures; the mixture of the two fractions is advisable.

The use of a standard addition calibration method to quantify the analytes in a young, aged 1 year and aged 2 year wine reveal that the wine matrix provides higher concentrations than the correct ones when a conventional calibration is made. The use of a matrix-standard calibration method with synthetic wine extracts spiked with the analytes is a less tedious way of decreasing the quantitative errors in routine analysis, but the influence of the matrix is not completely avoided.

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