JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Optimization of a Solid-Phase Extraction Method Using Copolymer Sorbents for Isolation of Phenolic Compounds in Red Wines and Quantification by HPLC

SILVIA PÉREZ-MAGARIÑO,* MIRIAM ORTEGA-HERAS, AND ESTELA CANO-MOZO

Estación Enológica, Instituto Tecnológico Agrario de Castilla y León (ITACyL), C/Santísimo Cristo 16, 47490 Rueda (Valladolid), Spain

Different solid-phase extraction (SPE) cartridges for the isolation of most of the phenolic compounds present in wines in low concentration were assayed and compared with the C18 SPE sorbent, which is the most common sorbent used for the isolation of these compounds. The use of C18 cartridges has several disadvantages; therefore, a SPE method using copolymer cartridges was developed, optimized, and validated. The method proposed seems to be a good alternative to replace C18 cartridges for the isolation of wine phenolic compounds. The advantages of the proposed SPE method with the HLB cartridge are that interferences can be eliminated with water without losing the compounds of great interest; the method has very good repeatability, reproducibility, and high percentages of recovery; it has a higher sensitivity and loading capacity than silica-based C18 cartridges due to the larger surface area of this type of sorbent, and the method is not adversely affected by drying, being more reproducible than C18 cartridge.

KEYWORDS: Phenolic compounds; extraction; cartridges; wines

INTRODUCTION

Phenolic compounds are important components of grapes and essential to wine quality. They are responsible for some sensorial characteristics of red wines and may play an important role in the health benefits attributed to moderate wine consumption.

Wine phenolic compounds can be classified in two groups, nonflavonoid and flavonoid. Nonflavonoid compounds include mainly simple phenolic alcohols, aldehydes, acids and their derivatives, and other phenols such as stilbenes. Flavonoid composition is mainly anthocyanins and flavanols, along with smaller amounts of flavonols and dihydroflavonols (*1*).

Anthocyanins and proanthocyanidins are the most studied compounds in wines, probably due to their major importance in wine characteristics and their strong presence or concentration. The reactions of anthocyanins and proanthocyanidins give rise to new pigments that play an important role in organoleptic changes taking place during wine aging. The color properties of these new wine pigments have been studied and compared to those of their anthocyanin precursors (*2*−*5*). The influence of proanthocyanidins on wine taste, mainly astringency and bitterness, has also been investigated (*6, 7*).

However, there are other phenolic compounds such as phenolic acids, flavonols, and stilbenes that, despite their low concentrations, are also interesting and important for wine quality (*8, 9*) and health benefits (*10, 11*).

Phenolic acids and flavonols play an important role in determining the sensorial characteristics of wines. Some of these compounds can act as cofactors, also called copigments, stabilizing the color of anthocyanins (*12, 13*). Different studies

Table 1. Different Solid-Phase Extraction Sorbents Used

commercial name	sorbent type	av particle size/amount of sorbent	supplier
XAD-2	Amberlite	150−250 μm/5 g	Sigma-Aldrich
C18	C18 silane	50 μm/500 mg	Isolute
C18(EC)	C18 silane and trimethylsilyl groups	50 μm/500 mg	Isolute
MFC18	monofunctional C18	50 μm/500 mg	Isolute
C8	C8 silane	50 μm/500 mg	Isolute
C8(EC)	C8 silane and trimethylsilyl groups	50 μm/500 mg	Isolute
PH	phenylsilane	50 μm/500 mg	Isolute
101	polystyrene−divinylbenzene copolymer	60 μm/500 mg	Isolute
ENV+	hydroxylated polystyrene−divinylbenzene copolymer	90 μm/500 mg	Isolute
HLB	*N*-vinylpyrrolidone−divinylbenzene copolymer	30 μm/200 mg	Waters
MCX	mixed mode cation-exchange and reversed-phase (*N*-vinylpyrrolidone−divinylbenzene copolymer)	30 μm/150 mg	Waters

* Corresponding author (telephone +34 983 868149; fax +34 983 868412; e-mail permagsi@itacyl.es).

10.1021/jf802100j CCC: $40.75
Published on Web 12/02/2008

Table 2. Peak Number, Retention Time, UV−Vis Data, and Quantification Wavelength of the Phenolic Compounds[a]

peak	compound	retention time (min)	UV−vis max (nm)	quantification wavelength (nm)
1	gallic acid	12.6	270	280
2	protocatechuic acid	23.2	260−294	280
3	*trans*-caftaric acid	28.6	298 (sh), 328	320
4	*cis*-coutaric acid	38.1	310	320
5	tyrosol	39.1	276	280
6	*trans*-coutaric acid	40.2	314	320
7	(+)-catechin	43.5	278	280
8	vanillic acid	48.6	260−292	280
9	*trans*-fertaric acid	49.1	298 (sh), 326	320
10	*trans*-caffeic acid	49.9	298 (sh), 322	320
11	hexose ester of *trans-p*-coumaric acid (1)	51.4	294 (sh), 310	320
12	syringic acid	55.2	274	280
13	hexose ester of *trans-p*-coumaric acid (2)	56.4	294 (sh), 310	320
14	(−)-epicatechin	58.0	278	280
15	ethyl gallate	63.5	272	280
16	*trans-p*-coumaric acid	66.1	298 (sh), 310	320
17	*cis-p*-coumaric acid	69.8	296	280
18	myricetin-glycosides	68.3	262 (sh), 302 (sh), 354	360
19	myricetin-glycosides	69.7	262 (sh), 302 (sh), 354	360
20	myricetin-glycosides	70.8	262 (sh), 302 (sh), 354	360
21	*trans*-resveratrol-3-glucoside	76.1	306−318	320
22	ellagic acid	78.5	368	370
23	quercetin-glycosides	80.9	264 (sh), 354	360
24	quercetin-glycosides	81.9	264 (sh), 354	360
25	quercetin-glycosides	84.2	264 (sh), 354	360
26	tryptophol	91.0	280	280
27	syringetin-3-glucoside	97.9	264 (sh), 356	360
28	myricetin	99.9	266 (sh), 304 (sh), 372	360
29	*cis*-resveratrol-3-glucoside	105.6	284	280
30	*trans*-resveratrol	108.0	306−316	320
31	quercetin	130.9	254 (sh), 372	360
32	kaempferol	135.9	264 (sh), 366	360
33	isorhamnetin	136.3	254 (sh), 370	360

[a] sh, shoulder.

have shown that increasing the concentration of cofactors leads to color intensification (hyperchromic shift) and also to a bathochromic shift in the wavelength of maximum absorbance, providing a blue shift from the formation of copigment complexes (*8, 14, 15*). A review (*8*) shows that cinnamic acids and quercetin glycosides are the principal compounds involved in copigmentation processes with anthocyanins, which affect the color of red wines.

Furthermore, flavonols and phenolic acids also influence certain characteristics, such as astringency and bitterness (*8, 9*). Hufnagel and Hoffmann (*9*) have shown that some flavonols such as quercetin-3-galactoside and syringetin-3-glucoside present a low taste threshold for velvety astringency and some phenolic acids such as caffeic and caftaric acids present a low threshold for puckering astringency.

Stilbenes are other phenolic compounds that have recently attracted great interest for their potentially beneficial effects on human health (*10, 11*).

Analysis of phenolic compounds from wine is rather complicated as they are so numerous and have different structures, which make it necessary to separate, fractionate, and/or concentrate them first to analyze them. Some authors have evaluated the phenolic compounds by direct injection of a wine sample in HPLC, without any preparation step (*16−23*). However, in these studies, all of the phenolic compounds could not be evaluated at the same time. Most researchers evaluate anthocyanins by direct wine injection. However, if the analysis of other phenolic compounds present in wines in lower concentration is desired, prior steps to purify, separate, and/or concentrate by liquid−liquid (L-L) or solid-phase extractions (SPE) should be carried out. In general, L-L extraction is a tedious, highly time-consuming process with high solvent costs and low recoveries (*24*).

Nowadays, SPE is becoming more used because it is rapid, economical, and sensitive and because different cartridges with a great variety of sorbents can be used (*25−29*). In addition, it can now also be automated, reducing processing time. C18 cartridges have been the most widely used in phenolic compound separation, but they have some disadvantages, such as low recovery for some compounds (such as highly polar compounds and hydroxybenzoic and hydroxycinnamic acids and their derivatives); care must also be taken to avoid drying the conditioned sorbent because its efficiency is reduced (*24, 30*). In addition, alcohol is often removed from the sample (*25, 26, 28, 31*), because alcohol reduces the retention of some phenolic compounds by the sorbent during the loading and washing phases, especially hydroxybenzoic acids. This reduces their

Table 3. Calibration Parameters for the Quantification of Phenolic Compounds

compound	concn range (mg/L)	R^2	response factor (mg/area units)	detection limit (mg/L)	quantification limit (mg/L)
hydroxybenzoic acids and derivatives					
gallic acid	0.27−72	0.9998	1.9 E−07	0.108	0.359
protocatechuic acid	0.11−28	0.9999	2.4 E−07	0.010	0.032
vanillic acid	0.11−28	0.9999	3.1 E−07	0.028	0.094
syringic acid	0.60−120	0.9999	2.2 E−07	0.137	0.456
ethyl gallate	0.20−52	1.0000	2.3 E−07	0.034	0.114
ellagic acid	0.18−1.2	0.9938	8.8 E−08	0.060	0.201
hydroxycinnamic acids					
trans-caffeic acid	0.96−64	0.9997	1.0 E−07	0.207	0.690
trans-p-coumaric acid	0.12−32	1.0000	9.6 E−08	0.025	0.085
stilbenes					
trans-resveratrol	0.11−28	0.9991	4.9 E−08	0.015	0.050
phenolic alcohols					
tyrosol	0.48−128	0.9999	8.9 E−07	0.033	0.111
tryptophol	0.42−112	0.9999	3.0 E−07	0.035	0.117
flavanols					
(+)-catechin	0.39−104	0.9999	8.6 E−07	0.036	0.120
(−)-epicatechin	0.42−112	0.9999	7.3 E−07	0.102	0.339
flavonols					
myricetin	2.50−40	0.9900	1.4 E−07	0.876	2.919
quercetin	1.12−15	0.9960	2.4 E−07	0.256	0.855
kaempferol	0.65−7.5	0.9997	9.1 E−08	0.087	0.289

Table 4. Phenolic Compounds Identified in the Water Fraction of Each Cartridge[a]

compound	XAD-2	C18	C18(EC)	MFC18	C8	C8(EC)	PH	101	ENV+	HLB	MCX
hydroxybenzoic acids and derivatives											
gallic acid	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX			
protocatechuic acid	XX	XXX	XXX	XXX	XXX	XXX	XXX	XXX			
vanillic acid											
syringic acid				XX							
ethyl gallate											
ellagic acid											
hydroxycinnamic acids and derivatives											
trans-caffeic acid			XX								
trans-p-coumaric acid											
cis-p-coumaric acid											
trans-caftaric acid		XX	XXX	X	XX	XX	XXX	X			
cis-coutaric acid			XX								
trans-coutaric acid		X	XX	X	X	XX	XX	X			
trans-fertaric acid											
hexose ester of *trans-p* coumaric acid (1)			XX								
hexose ester of *trans-p*-coumaric acid (2)											
phenolic alcohols											
tyrosol		XX	XXX	X	XX	XXX		X			
tryptophol											
flavanols											
(+)-catechin		X	XX	X							
(−)-epicatechin											

[a] X, trace amounts of this compound appeared; XX, the amount of this compound was distributed in two fractions; XXX, the main amount of this compound appeared in this fraction.

Table 5. Phenolic Compounds Identified in Diethyl Ether Fraction of Each Cartridge[a]

compound	XAD-2	C18	C18(EC)	MFC18	C8	C8(EC)	PH	101	ENV+	HLB	MCX
hydroxybenzoic acids and derivatives											
gallic acid	X	X	X	X	X	X	X	X	XXX		XX
protocatechuic acid	XX	X	X	X				X	XXX		XX
vanillic acid	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XX	XXX
syringic acid	XXX	XXX	XX	XXX	XXX	XXX	XXX	XXX	XXX	XX	XXX
ethyl gallate	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	X	XXX
ellagic acid	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XX		
hydroxycinnamic acids and derivatives											
trans-caffeic acid	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX		XX
trans-p-coumaric acid	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	X	XX
cis-p-coumaric acid	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX		XXX
trans-caftaric acid	XXX	XX	X	XXX	XX	XX	X	XXX	XX		X
cis-coutaric acid	XXX	XXX	XX	XXX	XXX	XXX	XXX	XXX	XXX		X
trans-coutaric acid	XXX	XXX	XX	XXX	XXX	XX	XX	XXX	XX		X
trans-fertaric acid	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XX		X
hexose ester of *trans-p* -coumaric acid (1)			X		X			X			
hexose ester of *trans-p*-coumaric acid (2)			X								
stilbenes											
trans-resveratrol-3-glucoside		XX	XX		XXX	XXX					
cis-resveratrol-3-glucoside		XX	XXX	XXX	XXX	XXX	XXX	XX			
trans-resveratrol	XXX	XXX	XXX	XXX	XXX	XXX	XX	XXX	XXX		
phenolic alcohols											
tyrosol	XXX	XX	X	XXX	XX	X	XXX	XXX	XXX	XX	XXX
tryptophol	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XX	XXX
flavanols											
(+)-catechin	XXX	XXX	XX	XXX	XXX	XX	XX	XX	X		
(−)-epicatechin	XXX	XXX	XXX	XXX	XXX	XXX	XX	XX			
flavonols											
myricetin	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX		X
quercetin	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX		XX
kaempferol	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX		XX
isorhamnetin	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX		XX
myricetin-glycosides		X	X			XX	X	X			
quercetin-glycosides		XX	XX	X	X	XX	X	X			
syringetin-3-glucoside		X	X			X					

[a] X, trace amounts of this compound appeared; XX, the amount of this compound was distributed in two fractions; XXX, the main amount of this compound appeared in this fraction.

recoveries if these two fractions are not collected. Other authors have also used XAD-2 resins as the sorbent to fractionate the phenolic compounds (*32*−*34*), low molecular weight phenols, and flavanols being quantified. However, the most polar compounds such as gallic and protocatechuic acid were lost in the washing phase.

Table 6. Phenolic Compounds Identified in Ethyl Acetate Fraction of Each Cartridge[a]

compound	XAD-2	C18	C18(EC)	MFC18	C8	C8(EC)	PH	101	ENV+	HLB	MCX
hydroxybenzoic acids and derivatives											
gallic acid									X	XX	XX
protocatechuic acid										XXX	XX
vanillic acid											XX
syringic acid											XX
ethyl gallate										XXX	
ellagic acid	X								XX	X	XX
hydroxycinnamic acids and derivatives											
trans-caffeic acid									X	XXX	XX
trans-p-coumaric acid					X				X	XXX	XX
cis-p-coumaric acid										XXX	
trans-caftaric acid	X								XX	X	XXX
cis-coutaric acid									X	XX	XXX
trans-coutaric acid	X								XX	XX	XXX
trans-fertaric acid									XX	XXX	XXX
hexose ester of *trans-p*-coumaric acid (1)	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	X	X
hexose ester of *trans-p*-coumaric acid (2)	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	X	X
stilbenes											
trans-resveratrol-3-glucoside	XXX	XX	XX	XXX		X	XXX	XXX	XXX		X
cis-resveratrol-3-glucoside	XXX	XX						XX	XXX		XX
trans-resveratrol							XX			XX	XX
phenolic alcohols											
tyrosol									X	XX	X
tryptophol									X	XX	X
flavanols											
(+)-catechin	X					XX	XX	XX	XXX		XX
(−)-epicatechin	X		X	X	X	X	XX	XX	XXX		XX
flavonols											
myricetin	X								X	X	XX
quercetin	X								X	XX	XX
kaempferol									X	XX	XX
isorhamnetin									X	XX	XX
myricetin-glycosides	XXX	XXX	XXX	XXX	XXX	XX	XXX	XXX	XXX		X
quercetin-glycosides	XXX	XX	XX	XXX	XXX	XX	XXX	XXX	XXX		X
syringetin-3-glucoside	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX		X

[a] X, trace amounts of this compound appeared; XX, the amount of this compound was distributed in two fractions; XXX, the main amount of this compound appeared in this fraction.

Table 7. Phenolic Compounds Identified in Methanol Fraction of Each Cartridge[a]

compound	HLB	MCX
hydroxybenzoic acids and derivatives		
gallic acid	XX	
protocatechuic acid		
vanillic acid		
syringic acid		
ethyl gallate		
ellagic acid	XXX	XX
hydroxycinnamic acids and derivatives		
trans-caffeic acid	X	
trans-p-coumaric acid	X	
cis-p-coumaric acid		
trans-caftaric acid	XXX	X
cis-coutaric acid	XX	
trans-coutaric acid	XX	X
trans-fertaric acid	X	
hexose ester of *trans-p*-coumaric acid (1)	XXX	XXX
hexose ester of *trans-p*-coumaric acid (2)	XXX	XXX
stilbenes		
trans-resveratrol-3-glucoside	XXX	XXX
cis-resveratrol-3-glucoside	XXX	XX
trans-resveratrol	XX	XX
phenolic alcohols		
tyrosol		
tryptophol		
flavanols		
(+)-catechin	XXX	XX
(−)-epicatechin	XXX	XX
flavonols		
myricetin	XXX	XX
quercetin	XX	XX
kaempferol	XX	
isorhamnetin	XX	
myricetin-glycosides	XXX	XXX
quercetin-glycosides	XXX	XXX
syringetin-3-glucoside	XXX	XXX

[a] X, trace amounts of this compound appeared; XX, the amount of this compound was distributed in two fractions; XXX, the main amount of this compound appeared in this fraction.

Therefore, the aim of this study was to assay different SPE cartridges for the isolation of most of the phenolic compounds present in wines in low concentration (such as simple phenolic acids and alcohols, flavonols, stilbenes, and their derivatives) and to compare them with the C18 SPE sorbent, which is the most common sorbent used for the isolation of these compounds. In addition, a SPE method was developed, with the selected cartridge being optimized and validated. The quantification of these phenolic compounds was carried out by HPLC. This work focuses on low molecular weight phenolic compounds, but the SPE cartridges or resins can also be used for the isolation of polymeric polyphenol fractions (*29, 35, 36*).

MATERIALS AND METHODS

Chemicals. Gallic acid, protocatechuic acid, vanillic acid, syringic acid, ellagic acid, *trans*-caffeic acid, *trans-p*-coumaric acid, tyrosol, tryptophol, (+)-catechin, myricetin, and kaempferol were purchased from Fluka (Buchs, Germany); *trans*-resveratrol was from Sigma-Aldrich (St. Louis, MO); and ethyl gallate, (−)-epicatechin, quercetin, and syringetin-3-glucoside were from Extrasynthèse (Lyon, France). Milli-Q water (Millipore, Bedford, MA), acetic acid (Panreac, Madrid, Spain), and methanol and acetonitrile (Laboratory-Scan, Dublin, Ireland) were used in high-performance liquid chromatography (HPLC) analyses.

Wine Samples. Different young and aged red wines were used for this study. The young wines were bottled just at the end of the malolactic fermentation and analyzed at <6 months in the bottle, and the aged wines were aged in barrels for 12 months. Wine samples were centrifuged at 3500 rpm for 5 min prior to any extraction.

Solid-Phase Extraction Procedures. Ten different sorbents in SPE cartridges and XAD-2 resin were assayed and compared with the C18 sorbent most used for the isolation of the phenolic compounds in wine (**Table 1**).

Table 8. Percentage of Extraction Efficiency of the Different Phenolic Compounds (Average of Three Cartridges) Using the Sorbents Studied with Respect to the C18

compound	XAD-2	C18(EC)	MFC18	C8	C8(EC)	PH	101	ENV+	HLB	MCX
hydroxybenzoic acids and derivatives										
gallic acid	***−13***	5	3	17	11	9	14	20	24	2
protocatechuic acid	41	1	1	***−22***	***−3***	15	3	2	31	10
vanillic acid	32	***−44***	69	102	7	39	83	120	147	145
syringic acid	18	123	118	154	61	166	176	147	329	223
ethyl gallate	4	***−50***	23	23	***−20***	9	31	18	83	79
ellagic acid	***−8***	***−46***	0	3	***−7***	***−7***	***−2***	***−6***	244	200
hydroxycinnamic acids and derivatives										
trans-caffeic acid	37	***−24***	46	48	***−34***	***−49***	53	61	199	156
trans-p-coumaric acid	***−10***	***−44***	1	9	***−1***	***−4***	8	11	124	140
cis-p-coumaric acid	***−13***	***−68***	***−13***	***−30***	***−27***	***−49***	***−6***	***−18***	78	77
trans-caftaric acid	157	50	159	145	7	0	141	217	287	240
cis-coutaric acid	176	40	199	146	***−34***	***−16***	199	245	678	611
trans-coutaric acid	74	10	83	80	***−1***	7	83	94	213	176
trans-fertaric acid	83	***−48***	95	100	***−4***	88	96	118	389	316
hexose ester of *trans-p*-coumaric acid (1)	99	***−10***	257	197	***−13***	***−19***	173	261	131	269
hexose ester of *trans-p*-coumaric acid (2)	119	***−6***	248	153	***−13***	***−23***	101	233	129	339
stilbenes										
trans-resveratrol-3-glucoside	***−5***	***−35***	19	***−68***	***−13***	***−7***	***−2***	15	67	26
cis-resveratrol-3-glucoside	20	***−48***	***−36***	***−15***	***−12***	***−20***	3	***−18***	171	113
trans-resveratrol	***−19***	***−39***	***−27***	***−26***	***−21***	59	***−35***	***−52***	65	82
phenolic alcohols										
tyrosol	7	***−9***	13	20	***−23***	***−92***	15	36	48	31
tryptophol	***−13***	***−40***	***−14***	***−17***	***−23***	***−24***	***−24***	***−34***	3	16
flavanols										
(+)-catechin	126	***−34***	117	17	***−56***	43	95	103	651	629
(−)-epicatechin	10	***−52***	159	75	***−46***	8	91	49	246	311
flavonols										
myricetin	***−8***	***−43***	***−25***	***−30***	***−25***	***−31***	***−34***	***−41***	78	72
quercetin	***−9***	***−31***	***−27***	***−29***	***−22***	***−24***	***−27***	***−34***	94	79
kaempferol	***−1***	***−48***	***−40***	***−46***	***−40***	***−39***	***−48***	***−52***	54	13
isorhamnetin	***−6***	***−42***	***−35***	***−40***	***−34***	***−35***	***−39***	***−42***	45	25
myricetin-glycosides	***−4***	***−34***	24	20	1	19	10	***−37***	34	11
quercetin-glycosides	***−4***	***−25***	12	6	2	5	5	***−12***	52	31
syringetin-3-glucoside	***−17***	***−12***	10	10	7	9	***−7***	***−27***	56	12

All cartridges were conditioned by rinsing with 3 mL of methanol and 3 mL of water. The wine sample to be extracted (1 mL) was acidified with 1 N H_2SO_4 (0.25 mL) prior to loading onto the conditioned cartridge. All cartridges were washed with 5 mL of Milli-Q water. The retained phenolic compounds were eluted with 5 mL of diethyl ether, followed by 5 mL of ethyl acetate. Then the cartridges were washed with 5 mL of methanol. All fractions were collected separately to ascertain whether some of the phenolic compounds were eluted during the washing step. The fractions were evaporated also separately to dryness in a rotary vacuum evaporator ($T < 35$ °C) and immediately dissolved in a known volume (1 mL) of a solution of methanol/water (20:80). A manifold system (Waters, Barcelona, Spain) was used for SPE.

Column separation on Amberlite XAD-2 resin (Sigma-Aldrich, St. Louis, MO) with a particle size of 150−250 μm was performed according to the method of Di-Stefano and Cravero (*32*) with certain slight modifications (*37*). The same solvents, water, diethyl ether, ethyl acetate, and methanol, were used, and the same procedure previously explained was carried out.

To validate the final optimized method, repeatability and reproducibility were evaluated using two different red wines (one young and one aged in barrels during 12 months) three times a day on three different days and by different analysts. Recoveries of the phenolic compounds were also calculated in the two red wines, and they were determined by adding 50 and 100 μL of a standard mixture to 2.5 mL of a wine sample. The two wines were spiked with known amounts of the compounds studied at two different concentrations. The standard mixture consisted of 16 phenols, because the rest of the compounds evaluated are not commercially available. Each wine for each concentration added was analyzed twice a day on two different days. **Table 10** shows the concentration of the different compounds added to the wines.

High-Performance Liquid Chromatography Diode Array Detection (HPLC-DAD) Analyses of Phenolic Compounds. The extracts obtained were analyzed with an Agilent Technologies LC series 1100, with a diode array detection system. Each extract was analyzed separately and previously filtered through PVDF filters with a pore size of 0.45 μm (Symta, Madrid, Spain). The injection volume was 100 μL for each fraction. The chromatographic separation was carried out on a reverse-phase Zorbax SB-C18 column (250 mm × 4.6 mm i.d., 3.5 μm particle size) provided by Agilent and thermostated at 25 °C. The chromatographic conditions were modified, on the basis of the method proposed by Monagas et al. (*38*). The solvents were (A) water/acetic acid (98:2) and (B) water/acetonitrile/acetic acid (78:20:2). The gradient was linear at a flow rate of 0.6 mL/min from 0 to 25% solvent B for 25 min, from 25 to 70% B for 35 min, and from 70 to 100% B for 40 min, then isocratic for 20 min, followed by washing with methanol and re-equilibration of the column for 10 min. Diode array detection was performed from 200 to 400 nm.

The phenolic compounds analyzed were identified by comparing their retention times and UV−vis spectra with their respective standard or with published data (*39*−*41*).

The quantification of the different phenolic compounds was carried out at different wavelengths (**Table 2**), applying each calibration line constructed using the corresponding standard. The calibration data are shown in **Table 3**. As can be seen, the linearity of all compounds is satisfactory with R^2 values >0.9990, except for ellagic acid, myricetin, and quercetin with R^2 values of 0.9938, 0.99, and 0.996, respectively. Furthermore, the linear ranges include the normal concentrations of these compounds in wines. Noncommercial available compounds were quantified using the calibration curves belonging to the most similar compounds: *trans-p*-coumaric for *cis-p*-coumaric acid, *cis*- and *trans*-coutaric acid, and hexose esters of *trans-p*-coumaric acid; *trans*-caffeic

Table 9. Percentage of Differences of the Different Phenolic Compounds (Average of Three Cartridges) Evaluated between the Method Proposed in This Study Using HLB Cartridge and the Methods Proposed by Del-Álamo et al. (*27*) (Method A) and Castillo-Muñoz et al. (*41*) (Method B)

compound	method A	method B
hydroxybenzoic acids and derivatives		
gallic acid	−1.9	−6.9
protocatechuic acid	−23.2	−1.2
vanillic acid	−5.8	1.3
syringic acid	−10.2	−4.3
ethyl gallate	nd[a]	8.2
ellagic acid	0.1	6.0
hydroxycinnamic acids and derivatives		
trans-caffeic acid	4.2	10.8
trans-p-coumaric acid	1.7	5.7
cis-p-coumaric acid	nd	nd
trans-caftaric acid	−3.5	−1.5
cis-coutaric acid	15.1	−3.1
trans-coutaric acid	−4.9	−5.0
trans-fertaric acid	nd	5.8
hexose ester of *trans-p*-coumaric acid (1)	−5.0	−11.5
hexose ester of *trans-p*-coumaric acid (2)	−13.3	−7.2
stilbenes		
trans-resveratrol-3-glucoside	−12.0	−3.4
cis-resveratrol-3-glucoside	5.1	−10.2
trans-resveratrol	−97.2	56.4
phenolic alcohols		
tyrosol	5.7	8.5
tryptophol	22.9	18.7
flavanols		
(+)-catechin	11.4	15.0
(−)-epicatechin	−12.0	9.1
flavonols		
myricetin	21.6	31.8
quercetin	11.1	30.3
kaempferol	52.3	62.7
isorhamnetin	39.1	45.6
myricetin-glycosides	−0.5	−3.7
quercetin-glycosides	5.4	−9.6
syringetin-3-glucoside	1.1	−35.2

[a] nd, compound not detected.

acid for *trans*-caftaric acid and *trans*-fertaric acid; *trans*-resveratrol for *trans*- and *cis*-resveratrol-3-glucoside; and flavonol aglycones for the respective flavonol glycoside derivatives.

The concentrations of each phenolic compound in each fraction (water, diethyl ether, ethyl acetate, and methanol) for each cartridge were summed to obtain the total concentrations.

RESULTS AND DISCUSSION

Cartridge Comparison. The solvents diethyl ether (DE), ethyl acetate (EA), and methanol were selected because they are the ones used most (*31*) and because they have the ability to elute phenolic compounds. However, it should be also pointed out that after the elution with the three solvents, the cartridges presented a dark purple color, which could mean that some polymeric polyphenols are retained in the cartridge.

Tables 4−**7** show the phenolic compounds identified in each fraction and cartridge studied.

As can be seen, only the copolymer sorbents (ENV+, HLB, and MCX), with the exception of the 101 cartridge, were able to retain the phenolic compounds, even the most polar ones (**Table 4**). In the other cartridges and in the XAD-2 resin, the gallic and protocatechuic acids were especially lost in the washed fraction (water). Some hydroxycinnamic acids and phenolic alcohols were also lost in this fraction, in different amounts depending on the type of cartridge. The cartridges with trimethylsilyl groups presented the worst phenol retention, also losing tartaric esters of hydroxycinnamic acids, tyrosol, and catechin. All of the cartridges and XAD-2 resin were able to retain the stilbenes and flavonols because they are less polar compounds.

The DE fraction (**Table 5**) obtained with the XAD-2 resin, the nonpolymer sorbents, and the 101 cartridge mainly contained the hydroxybenzoic and hydroxycinnamic acids and their derivatives, with the exception of the hexose esters of *trans-p*-coumaric acid. In these fractions, stilbenes, phenolic alcohols, flavanols, and flavonol nonglycosides were also found. The hexose esters of *trans-p*-coumaric acid, stilbenes, and flavonol glycosides were collected in the EA fractions (**Table 6**).

The polymer sorbents showed different behaviors. The DE fraction of ENV+ also eluted the phenolic compounds found in the nonpolymer cartridges previously commented. The HLB cartridge showed higher retention of phenolic compounds, because the DE was only able to elute some amounts of vanillic and syringic acid, ethyl gallate, and phenolic alcohols, and it was necessary to add EA to elute most of the phenolic compounds obtained in the DE fraction with the other cartridges (**Table 5**). The MCX cartridge showed an effect halfway between ENV+ and HLB cartridges, the phenolic compounds being distributed between the DE and EA fractions (**Tables 5** and **6**).

In the methanol fraction phenolic compounds were detected in only HLB and MCX cartridges (**Table 7**), which included mainly ellagic acid, hydroxycinnamic derivatives, stilbenes, flavanols, and flavonols.

Therefore, despite the fact that the 101 cartridge is a polystyrene−divinylbenzene copolymer, and behavior more similar to the other cartridges with copolymer sorbent (ENV+, HLB and MCX) should be expected, the results obtained for this cartridge were more similar to those obtained for the nonpolymer sorbents.

Taking into account that interfering substances or nondesirable compounds such as sugars and acids (which can complicate the subsequent HPLC analysis) are also eliminated in the water fraction, the polymer sorbent cartridges (ENV+, HLB, and MCX) could be expected to be the most suitable one, because no losses in phenols were observed in this fraction.

The concentrations of the phenolic compounds extracted with the different cartridges studied (evaluated as the sum of the concentrations found in the different fractions, water, DE, EA, and methanol, for each cartridge) were compared with the concentrations obtained with the C18 cartridges and evaluated in the same way. The results obtained were expressed as percentage of extraction efficiency with respect to the C18 cartridge (**Table 8**), to select the most suitable cartridge. Negative percentages in bold indicate that the concentration of each compound evaluated from each cartridge is lower than the concentration found from the C18 cartridge. It can be observed that cartridges with trimethylsilyl groups gave lower values than the C18 cartridge in most of the phenolic compounds evaluated, which means that they are not suitable for phenol analyses. The PH cartridge did not show, in general, better results than the C18 sorbent.

The XAD-2 resin showed higher values in hydroxycinnamic derivatives and flavanols than the C18, the concentrations of the other phenols being similar.

The MFC18, C8, and 101 cartridges showed the same behavior. These cartridges generally achieved higher values than the C18 in hydroxycinnamic derivatives, vanillic and syringic acids, and flavonols, but lower values in stilbenes and flavonol nonglycosides. These results are in accordance with those

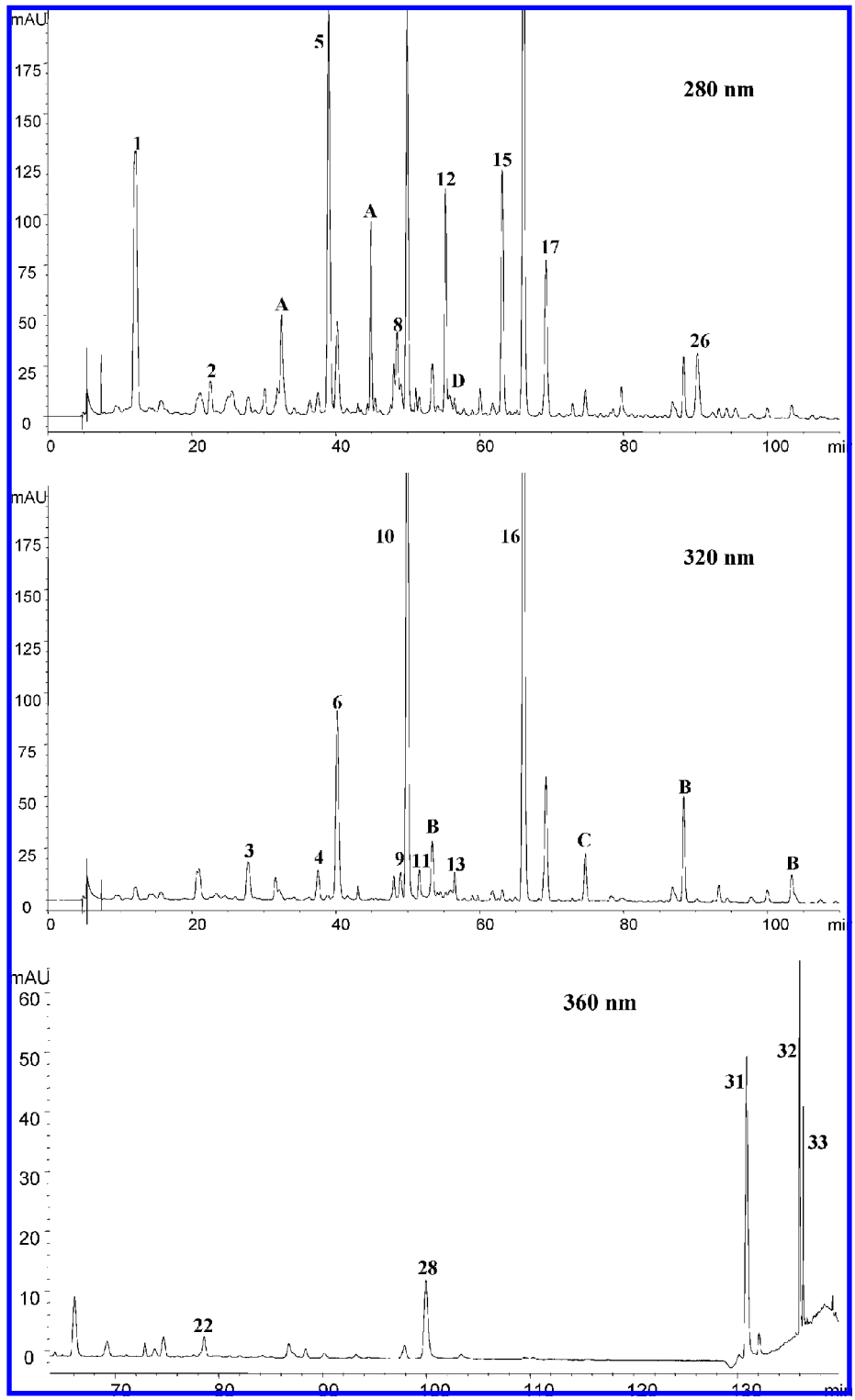

Figure 1. Chromatograms of the ethyl acetate fraction obtained by the SPE method proposed with an HLB cartridge of an aged red wine monitored at 280, 320, and 360 nm. Peak numbers are the compounds shown in **Table 2**; A−C, derivatives of gallic, *trans-p*-coumaric, and *trans*-caffeic acid, respectively; D, dihydroquercetin derivative.

obtained by other authors (*24, 26, 30*) who found that the C18 cartridge had low sensitivity, mainly for the hydroxycinnamic derivatives.

The polymer cartridge ENV+ showed results similar to those commented before for the MFC18. Lower values in flavonol glycosides than in the C18 cartridge were also detected.

The best results were obtained with *N*-vinylpyrrolidone−divinylbenzene copolymer sorbents, both HLB and MCX, giving the highest values of all the phenolic compounds studied, as can be seen in **Table 8**. They could thus be considered the most suitable for the extraction of the phenolic compounds studied.

In consideration of these results, two methods proposed by other authors who used the HLB and MCX cartridges were also assayed.

The first method, called "method A", was developed by Del-Álamo et al. (*27*). These authors optimized a SPE method using HLB to evaluate hydroxybenzoic and hydroxycinnamic acids

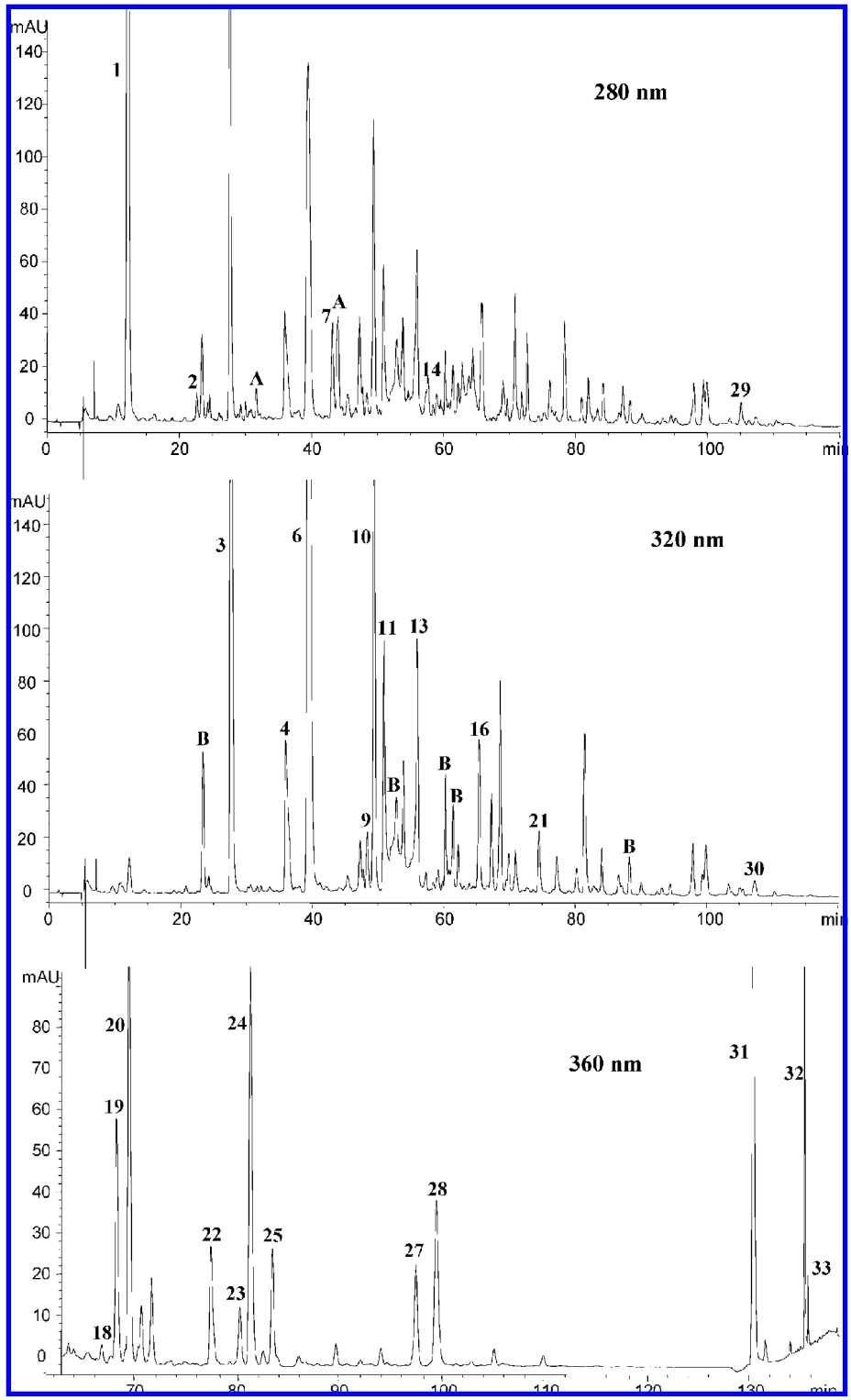

Figure 2. Chromatograms of the methanol fraction obtained by the SPE method proposed with an HLB cartridge of an aged red wine monitored at 280, 320, and 360 nm. Peak numbers are the compounds shown in **Table 2**; A and B, derivatives of gallic and *p*-coumaric, respectively.

and aldehydes. In this method the wine was concentrated to dryness and dissolved in a synthetic wine before being loaded onto the cartridge. After that, two solvents were used to elute the phenolic compounds, 5% of acetic acid in acetonitrile/water (10:90), followed by 2% of NH_3 in acetonitrile/water (10:90). These two fractions were collected in the same container and evaporated to dryness in a rotary vacuum evaporator, and the residue was dissolved in a solution of acetonitrile/water (20:80).

The second method, called "method B", was developed by González-Manzano et al. (*42*) and Castillo-Muñoz et al. (*41*), who employed MCX cartridges to separate proanthocyanidins and flavonols, respectively. Both research groups eluted these compounds with methanol, and the fraction obtained was evaporated to dryness in a rotary vacuum evaporator. The residue was dissolved in a solution of methanol/water (20:80).

In both methods A and B, the results obtained were generally good and similar to those found with our SPE method with HLB

Table 10. Reproducibility (Average of Nine Cartridges) and Percentages of Recovery (Average of Four Cartridges in Each Addition) Determined in Wines for the Phenolic Compounds Evaluated

	reproducibility (% RSD)		recovery					
			amounts added (mg)		recovery in young wine[b]		recovery in aged wine[b]	
compound	% RSD in young wine	% RSD in aged wine	add 1	add 2	add 1	add 2	add 1	add 2
hydroxybenzoic acids and derivatives								
gallic acid	7.2	4.5	0.0111	0.0222	68 (6.6)	77 (8.4)	89 (6.9)	84 (6.5)
protocatechuic acid	5.2	6.6	0.0025	0.0050	55 (5.8)	59 (2.7)	66 (2.9)	65 (5.8)
vanillic acid	5.2	6.6	0.0036	0.0072	79 (7.2)	84 (5.8)	90 (8.3)	89 (7.1)
syringic acid	3.7	3.6	0.0043	0.0085	97 (3.1)	99 (4.7)	99 (6.4)	99 (3.7)
ethyl gallate	3.3	3.4	0.0061	0.0122	90 (5.8)	95 (3.7)	94 (5.0)	93 (6.3)
ellagic acid	nd[a]	8.9	0.0029	0.0058	44 (2.0)	33 (2.9)	26 (7.3)	28 (4.2)
hydroxycinnamic acids and derivatives								
trans-caffeic acid	6.1	8.6	0.0082	0.0163	67 (8.7)	68 (8.2)	91 (7.6)	87 (8.1)
trans-p-coumaric acid	3.1	5.1	0.0050	0.0100	93 (4.4)	93 (5.7)	92 (5.7)	92 (4.1)
cis-p-coumaric acid	2.7	6.2						
trans-caftaric acid	5.8	4.6						
cis-coutaric acid	5.8	7.1						
trans-coutaric acid	7.0	6.9						
trans-fertaric acid	10.5	9.1						
hexose esters of *trans-p*-coumaric acid	6.9	2.5						
stilbenes								
trans-resveratrol-3-glucoside	7.5	6.4						
cis-resveratrol-3-glucoside	9.2	6.0						
trans-resveratrol	6.9	7.4	0.0012	0.0024	77 (8.7)	71 (9.0)	70 (1.2)	70 (4.5)
phenolic alcohols								
tyrosol	3.3	2.4	0.0452	0.0904	76 (7.1)	82 (3.0)	80 (7.5)	82 (2.9)
tryptophol	8.0	7.8	0.0101	0.0202	82 (5.0)	80 (8.1)	97 (5.4)	96 (3.4)
flavanols								
(+)-catechin	6.6	4.9	0.0216	0.0431	89 (6.2)	90 (5.5)	96 (5.5)	91 (7.7)
(−)-epicatechin	7.8	6.6	0.0215	0.0430	73 (3.4)	88 (8.0)	76 (3.6)	83 (4.7)
flavonols								
myricetin	9.2	8.6	0.0051	0.0103	27 (4.2)	33 (4.0)	26 (8.5)	37 (8.7)
quercetin	9.1	6.7	0.0006	0.0012	65 (6.1)	70 (6.3)	103 (7.2)	96 (8.1)
kaempferol	7.3	7.7	0.0010	0.0019	28 (5.9)	33 (4.3)	26 (6.9)	30 (7.3)
isorhamnetin	5.8	9.6						
myricetin-glycosides	3.0	7.0						
quercetin-glycosides	8.5	3.6						
syringetin-3-glucoside	7.1	6.4						

[a] nd, not detected. [b] Standard deviation is shown in parentheses.

and MCX cartridges because the percentage of differences was lower than 10% (**Table 9**). With methods A and B, worse results (negative percentages) were obtained in only some hydroxycinnamic acids, such as hexose esters of *trans-p*-coumaric acids and *cis-p*-coumaric acid, stilbenes, and flavonol glycosides. However, better results were found in nonglycoside flavonol compounds, especially with method B.

In the method proposed by Del-Álamo et al. (*27*), it is necessary to concentrate the wine to dryness, and the MCX cartridge does not show a significant improvement of the results of the phenolic compounds, these cartridges being more expensive. Considering the results indicated above and taking these two facts into account, we decided to optimize and validate the SPE method developed in our laboratory using HLB cartridges.

Optimization of the SPE Method. As has just been commented and in consideration of the results obtained, the final cartridge selected for better isolation of phenolic compounds was the HLB one, which has an *N*-vinylpyrrolidone−divinylbenzene copolymer as a sorbent. The solvents assayed to elute phenols were DE, EA, and methanol. However, as can be seen in **Table 5**, only six phenolic compounds were found in the DE fraction of HLB, which were also eluted with EA. Furthermore, they were not overlapped with other compounds in the EA fraction. Therefore, the first step in optimizing the SPE method was to eliminate the DE fraction, the first solvent used after washing with water being EA. The results obtained using DE and EA solvents and only the EA solvent were compared, and the differences in concentrations of the phenolic compounds found were lower than 5% (data not shown). It was therefore considered to be appropriate to eliminate the DE fraction. Then only two fractions (two solvents, EA and methanol) were needed to isolate and quantify the phenolic compounds in the final SPE method proposed.

Figures 1 and **2** show representative chromatograms of EA and methanol fractions, respectively, obtained by the SPE method proposed using HLB cartridges for an aged red wine. As can be seen, 33 low molecular weight phenolic compounds can be clearly quantified. Other phenols can also be quantified, although at this moment we are not able to identify them. The compounds marked A, B, and C showed spectra similar to gallic, *trans-p*-coumaric, and *trans*-caffeic acid, respectively, so they could be derivatives of these acids. The compound marked D showed a maximum absorbance at 292 nm, and according to the data shown in Hernández et al. (*43*), it could be a dihydroquercetin derivative.

The final method was repeated using HLB cartridges with higher capacity (500 mg of sorbent), loading 2.5 mL of wine previously acidified with 1 N H_2SO_4 (0.625 mL) and eluting with 12 mL of each solvent. After evaporation of the different solvents, the samples were dissolved in 1.5 mL of a solution of methanol/water (20:80). The results showed the same phenolic profiles; that is, the same phenolic compounds were identified. However, a higher concentration of the compounds was found, which facilitated the quantification of the compounds and reduced errors, improving the sensitivity of the method.

Therefore, if achieving a higher concentration of the phenolic compounds is needed, cartridges with higher capacity that can be loaded with higher wine volumes could be used.

Validation and Recovery of Phenolic Compounds for the Optimized Method. The reproducibility and recovery of the compounds were carried out using the HLB cartridge with higher capacity (500 mg). The average values of the relative standard deviation (RSD) are shown in **Table 10**. The coefficients of variation or percentage of RSD was no higher than 10% for reproducibility and was lower than 6% in most cases. This SPE method can therefore be considered to have good reproducibility.

Considering these good results in reproducibility, recoveries of the phenolic compounds were determined as has previously been commented under Materials and Methods. **Table 10** shows the concentration of the different compounds added to the wines, as well as the recovery for each compound.

In general, good recoveries were obtained. Only the flavonols, myricetin and kaempferol, and ellagic acid showed poor recoveries of around 30%. These results can be due to the fact that these compounds are very reactive and may react with other phenolic compounds. In addition, only the protocatechuic acid showed low recovery (average of 61%), the rest of the compounds having recoveries of >80%. These results are in agreement with those found by other authors (*26, 27, 44*). However, it should be pointed out that in those studies, the recoveries were calculated by additions on synthetic wines, and in our study the additions have been carried out on two different wines, the matrix effect then being taken into account. Only Russo et al. (*45*) evaluated the recoveries using commercial wines, although in that study most of the phenolic compounds evaluated were quantified by luminescence.

In summary, the use of C18 cartridges implies several disadvantages, such as low recoveries, the need to take care not to dry the cartridge after conditioning because this can give rise to low repeatability, and low detection of some phenolic compounds (such as hydroxycinnamic acids and their derivatives). Therefore, the polymer cartridge, mainly the HLB with *N*-vinylpyrrolidone−divinylbenzene copolymer as a sorbent, seems to be a good alternative to replace C18 cartridges for the isolation of wine phenolic compounds.

The advantages of the proposed SPE method with the HLB cartridge are that interferences can be eliminated with water without losing the compounds of great interest; the method has a higher sensitivity for the compounds slightly detected with the C18 cartridges; it has very good repeatability, reproducibility, and high percentages of recovery; it has a higher loading capacity than silica-based C18 cartridges due to the larger surface area of this type of sorbent; and the method is not adversely affected by drying, being more reproducible. The SPE method proposed allows quantification of at least 33 individual phenolic compounds, and it is a rapid technique because only organic fractions have to be concentrated in this method, reducing total sample preparation time. Therefore, with this method and the one proposed previously for the direct quantification of anthocyanins (*23*), both flavonoid and nonflavonoid phenolic compounds can be quantified. In addition, the HLB cartridges can be used to isolate the polymeric phenol compounds (*29*), so all of the phenolic fractions could be isolated and quantified.

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Received for review July 10, 2008. Revised manuscript received October 8, 2008. Accepted October 12, 2008. We thank the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) for funding provided for this study under Projects RTA2006-071 and RTA2007-074. S.P.-M. and M.O.-H. are also grateful to the INIA for partially financing their work contracts. E.C.-M. also thanks the Instituto Tecnológico Agrario de Castilla y León (ITACyL) for her technical grant.

JF802100J