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Analytical Methods

Solid cation exchange phase to remove interfering anthocyanins in the analysis of other bioactive phenols in red wine

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ABSTRACT

Bioactive phenols (BPs) are often targets in red wine analysis. However, other compounds interfere in the liquid chromatography methods used for this analysis. Here, purification procedures were tested to eliminate anthocyanin interference during the determination of 19 red-wine BPs. Liquid chromatography, coupled to a diode array detector (HPLC-DAD) and a mass spectrometer (UPLC-MS), was used to compare the direct injection of the samples with solid-phase extractions: reversed-phase (C18) and strong cation-exchange (SCX). The HPLC-DAD method revealed that, out of 13 BPs, only six are selectively analyzed with or without C18 treatment, whereas SCX enabled the detection of all BPs. The recovery with SCX was above 86.6% for eight BPs. Moreover, UPLC-MS demonstrated the potential of SCX sample preparation for the determination of 19 BPs. The developed procedure may be extended to the analysis of other red wine molecules or to other analytical methods where anthocyanins may interfere.

1. Introduction

Design of functional foods and beverages increasingly requires the examination of bioactive molecules (Bidlack & Wang, 2006; Khan, Grigor, Winger, & Win, 2013). Phenols, which are biosynthesized as part of the plant's defense system (Fig. 1), are often targets in the analysis of fruit-derived products (Crozier, Clofford, & Ashihara, 2006). Grapes and wines are potential sources of such compounds in the human diet (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). Natural phenols may be classified as nonpigmented (or sometimes, soft) or strongly pigmented molecules. Nonpigmented molecules provide more health benefits such as: cardiovascular protection, and antitumor and antioxidant activity (Barjot et al., 2007; Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012; Nigdikar, Williams, Griffin, & Howard, 1998; Renaud & De Lorgeril, 1992; Roupe, Remsberg, Yáñez, & Davies, 2006; Whelan, Sutherland, Mccormick, Yeoman, & Jong, 2004). Because of their high nutraceutical value, the nonpigmented compounds, called bioactive phenols (BPs), have received special attention in food research (Bidlack & Wang, 2006; Khan et al., 2013).

Technological advances in analytical instrumentation have enabled the analysis of several compounds that affect human health, including that of BPs in wines (Ribéreau-Gayon et al., 2006). High-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC), coupled to an ultraviolet/visible detector (UV/Vis), a diode array detector (DAD), or a mass spectrometer (MS), are the main techniques for BP analysis, since BPs are nonvolatile organic molecules (Cotea,

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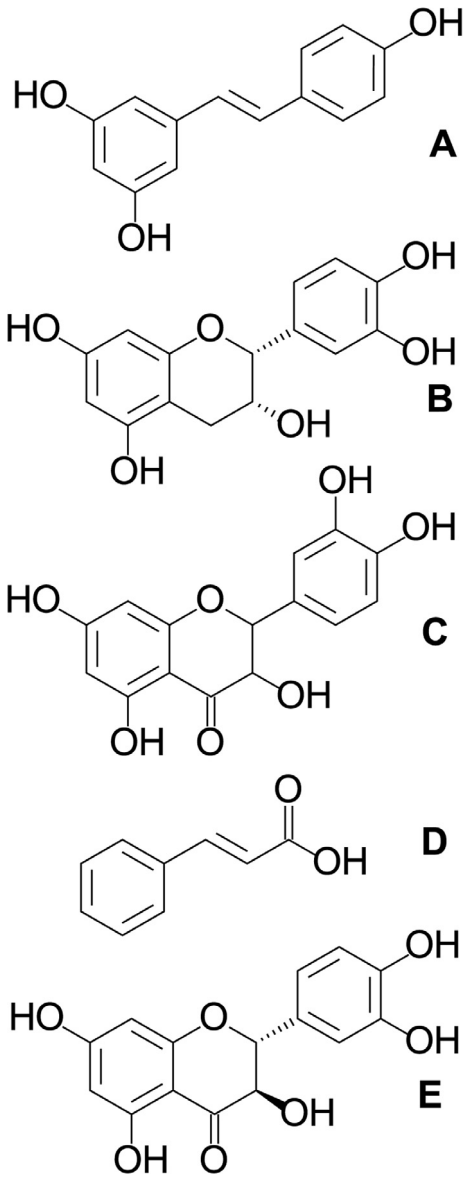

Fig. 1. Representative structures of the BP chemical classes: A) stilbenes: *trans*-resveratrol, B) flavanols: (-)-epicatechin, C) flavonols: quercetin, D) cinnamic acid derived: *trans*-cinnamic acid and E) flavanonol: taxifolin.

Luchian, Bilba, & Niculaua, 2012; Ignat, Volf, & Popa, 2011; Lorrain, Ky, Pechamat, & Teissedre, 2013; Pereira, Câmara, Cacho, & Marques, 2010; Rodríguez-Medina, Segura-Carretero, & Fernádez-Gutiérrez, 2009; Silva, Guerra, Foresti, & Bergold, 2016; Silva, Pereira, Woutera, Giróa, & Câmara, 2011; Tenore, Troisi, Di Fiore, Manfra, & Novellino, 2011). However, pigmented phenols, for example, may interfere with BP analysis in wines, as they have similar chemical properties (such as polarity), and as a result, they interact with the HPLC or UPLC columns fitted with most commonly used detectors (DAD and MS).

Another relevant aspect is the purification of the samples prior to the analysis in order to preserve the chromatographic columns, as they are usually expensive. Therefore, sample preparation is a crucial step for BP analysis in red wines through this instrumentation, and some attempts have been made to perform direct injection of the samples, a simple procedure, which, however, is not always selective and accurate (Pereira et al., 2010; Tenore et al., 2011) and only permits the analysis of a limited number of BPs (Silva et al., 2016). Other methods use solid-phase extraction (SPE) by different separation mechanisms or liquid-liquid extraction (Lorrain et al., 2013; Malovaná, Montelongo, Péreza, Rodríguez-Delgado, 2001), although the latter is more laborious and tends to give lower yields than SPE (Silva et al., 2011; Villiers, Lynen, Crouch, & Sandra, 2004). An SPE adsorbent commonly found in the literature for this purpose is octadecylsilane (C18), which is a reverse-phase-type adsorbent (Lorrain et al., 2013; Mattivi, 1993; Pérez-Magarino, Ortega-Heras, & Cano-Mozo, 2008). However, other types of adsorbents and techniques have also been proposed, such as anionic strong exchange (Figueiredo-González, Regueiro, Cancho-Grande, & Simal-Gándara, 2014; Guillén, Barroso, & Pérez-Bustamante, 1996; Guillén, Merello, Barroso, & Pérez-Bustamante, 1997), styrene-divinylbenzene (Silva et al., 2011; Villiers et al., 2004), molecularly imprinted polymers (Hashim et al., 2013), or mesoporous silica (Cotea et al., 2012).

In order to achieve suitable elimination of interferences from the wine matrix, it is important to identify these chemical differences with regard to BP analytes. Pigmented phenols are some of the major interferences; they are positively charged, whereas BPs are neutral in acidic media. Because of this observation, a strong ionic exchange mechanism was considered by Figueiredo-González et al. (2014), who used a strong anion exchange cartridge in which the reaction of anthocyanin with sodium bisulfite was necessary for retention by the adsorbent. In another report, a strong cationic exchange cartridge was used, although the study focused on the analysis of anthocyanins in fruits and vegetables and they probably discarded the BPs which interfere in their analyses (He & Giusti, 2011). Such processes could be simplified through the retention of positively charged molecules on a strong cationic exchange cartridge, whereas BPs could be directly collected for the analysis. Considering that "strong cation exchange linked to benzyl ring cartridges" (SCX) combine both ionic and hydrophobic interactions (Moldoveanu & David, 2002), SCX could be a selective option to eliminate positively charged organic molecules that interfere BP analysis. Therefore, this study aimed to compare the BP recovery and selectivity obtained after SCX treatment of some traditional sample preparations of red wines, using the most commonly used instrumentation for analytical purposes (HPLC-DAD and UPLC-MS).

2. Materials and methods

2.1. Chemicals and standards

Methyl alcohol (MeOH) and formic acid were HPLC grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagent grade ethyl alcohol (EtOH) and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Ultra-pure water (Milli-Q, Millipore, USA) was used to prepare all the solutions. Analytical standards of *trans*-resveratrol, *trans*-piceid, *trans*-ε-viniferin, quercetin, myricetin, kaempferol, rutin, *trans*-cinnamic acid, *p*-coumaric acid and *trans*-ferulic acid were from Sigma-Aldrich (St. Louis, MO, USA). (+)-Catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate, procyanidin B1, quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, and taxifolin were acquired from Extrasynthese (Genay Cedex, France). All the phenol standards were of at least 94% purity.

A stock solution comprising 200 mg/L of each phenol was diluted in EtOH 70% (v/v in water). Fractions of the stock solution were stored at 4 °C for a week. The working standard solutions were freshly prepared daily.

2.2. Samples of red wines

A commercial Cabernet Sauvignon wine (CS1) from Rio Grande do Sul, Brazil, was used to evaluate the effect of the matrix and to

determine a suitable methodology for sample preparation. The original wine sample was diluted 1.25 times with EtOH 70% (v/v), followed by filtration through a 0.22 μm polyvinylidene fluoride (PVDF) membrane. The sample was diluted to maintain the same proportion of the matrix in the fortified samples described below.

A fortified sample of CS1 was prepared by adding an appropriate volume of the stock solution so as to have a final concentration of 10 mg/L of each phenol. To obtain this, the matrix was diluted 1.25 times. The enriched sample was processed according to each optimization test (see Section 2.3).

The optimized sample purification method was applied to Merlot, Syrah, and Cabernet Sauvignon (CS1 and CS2) samples from Rio Grande do Sul. All the red wine samples were conserved at 18 ± 2 °C until analysis.

2.3. Sample preparation tests

Six different tests were conducted to evaluate the analytes according to each sample preparation: the traditional methods (direct injection and C18 SPE cartridge) and SCX SPE cartridges (SCXa, SCXb, SCXc, and SCXd tests). The SPE cartridges were coupled to a vacuum manifold (Supelco, USA).

Co-elution of the analytes with anthocyanins was considered in each test. A preliminary test of the direct injection of the CS1 sample was conducted using a validated HPLC-DAD method (Silva et al., 2016).

The purification of CS1 was tested by an RP tri-functional bonded (C18) cartridge, with 900 mg of sorbent and a particle size of 55–105 μm (tC18, Waters). First, the cartridges were conditioned with 10 mL of MeOH, 10 mL of water, and 3 mL of the sample. Any resulting waste was disposed of. Then, 2 mL of the sample were passed through the cartridge and the fraction was collected for subsequent HPLC-DAD injection.

Another test (SCXa) involved verifying the elimination of interferences using an SCX cartridge with 200 mg of sorbent and a particle size of 33 μm (Strata-XC, Phenomenex). After this, different experiments were conducted to determine the optimal conditions for sample purification (SCXb, SCXc, and SCXd tests). All cartridges were prepared beforehand by adding 1 mL of MeOH, followed by 1 mL of water. SCXa and SCXb tests involved passing 2 and 3 mL of the sample through the cartridges, respectively, and discarding the corresponding portions. The SCXa test required the use of 1 mL of MeOH with 0.1 M HCl for elution, whereas SCXb required 1 mL of MeOH/EtOH (90:10 v/v) with 0.1 M HCl. In the SCXc and SCXd tests, a portion of the sample (1 mL) was collected with the eluent, through elution with MeOH/EtOH (90:10 v/v) and 0.1 M HCl and with MeOH and 0.1 M HCl, respectively. The conditions and elution sequences for each test are summarized in Supplementary Table 1.

2.4. HPLC-DAD method

The phenol determinations were carried out through an HPLC-DAD method previously validated by our group for six BPs, as described below (Silva et al., 2016). This method was extended to analyze a further seven BPs (for a total of 13). Shimadzu system (Kyoto, Japan) equipped with a diode array detector (DAD) was used, managed by CLASS VP software (Shimadzu). The C8 column used (Vertical, 150 × 4.6 mm, 5 μm) was protected with a C8 guard column (Phenomenex). The flow rate was 1 mL/min and the injection volume was 20 μL. The detection was performed according to the maximum UV/Vis absorption (λ_{max}) of each analyte (Table 1). The aqueous eluent consisted of formic acid and water (1:1000, v/v), and the organic eluent consisted of MeOH, formic acid, and water (900:1:100, v/v). The linear gradient was: from 30% to 45% of B in 7 min, 45% of B over 7 min, from 45% to 55% of B in 1 min, from 55% to 65% of B in 9 min, from 65% to 90% of B in 1 min, 90% of B over 2 min, from 90% to 30% of B in 0.5 min, and 30% of B over 2.5 min. The chromatograms were recorded for 30 min.

Sample and standard solutions were injected in triplicate. All peak integrations were performed according to previously established conditions for the standard solutions, such as the retention time (R_T) and λ_{max}. Quantification was carried out using the external standard method and by comparing each analyte with its corresponding calibration curve.

The selectivity was also evaluated. The peak purity obtained for each analyte in the standard solutions, wine samples, and fortified samples was analyzed by DAD. Spectra were collected from 200 to 600 nm. The peaks were considered pure when the peak purity index was over 0.95, as calculated by CLASS VP software.

The linearity of Silva et al. (2016) was extended from six to 13 BPs using three calibration curves on different days, which involved injections in triplicate. The stock solution (Section 2.1) was used to prepare calibration curve solutions at six concentration levels (1.0, 5.0, 10.0, 20.0, 30.0, and 40.0 mg/L), which were diluted in EtOH 70% (v/v). The concentrations of cinnamic acid were 0.1, 1.0, 2.5, 5.0, 7.5, and 10.0 mg/L. The linear equations were calculated using the least squares method and the linearity was evaluated by ANOVA.

The accuracy was assessed by a recovery study. The analytes were tested by adding 10 mg/L of each standard to a red wine sample (CS1) in triplicate. The calculation of the recovery was carried out using the following formula: $R\%$ = [analyte in fortified sample] × 100/([analyte on original sample] + [analyte added]).

2.5. UPLC-MS method

The identifications of 19 BPs was performed on a Waters Acquity UPLC System (Milford, MA, USA), that comprised a quaternary pump, an autosampler, a column oven, a single mass quadrupole detector (MS), and Empower 3 software. A C18 BEH column was used (Waters, 50 × 2.1 mm, 5 μm), which was protected with a guard column of the same material (Waters). The flow rate was 0.5 mL/min and the injection volume was 1 μL. The detection was performed based on the molecular weight of each analyte (Table 1). The eluents were the same as those used for the HPLC-DAD method. The linear gradient was: from 30% to 65% of B in 2.5 min, from 65% to 90% of B over 0.5 min, 90% of B over 0.8 min, from 90% to 30% of B in 0.2 min, and 30% of B over 1.0 min. The chromatograms were recorded for 5 min.

MS detection was conducted on a Waters QDa instrument (Milford, MA, USA) with an electrospray (ESI) source. ESI mode was selected to obtain a high selectivity for each analyte. The probe temperature was 600 °C, the capillary voltage was −0.8 and +0.8 kV, the cone voltage was 15 V, and the sampling rate was 1 point across the peak. Acquisitions were obtained by scanning from m/z 140 to 615 Da.

3. Results and discussion

3.1. Evaluation of direct injection by HPLC-DAD

Thirteen BP standards were suitably separated and detected through the HPLC-DAD method, whose chromatograms were illustrated at 278 and 320 nm to cover all the detection signals (Fig. 2A). The linearity and selectivity (R_T and λ_{max}) obtained here and established previously (Silva et al., 2016) for each isolated analytical standard determined by the HPLC-DAD system are listed in Table 1. The method displayed good linearity for all BPs; their correlation factors (r) were up to 0.99, as corroborated by ANOVA

Table 1
HPLC-DAD and UPLC-MS parameters for the determination of bioactive phenols in red wines.

Analyte	HPLC-DAD[a]					UPLC-MS		
	a	b	*r*	R_T (min)	λ_{max} (nm)	R_T (min)	ESI	*m/z*[b]
Stilbenes								
trans-Resveratrol	−35370[c]	167034[c]	0.999[c]	15.3	306	1.39	+	229
trans-Piceid	−16082	84951	0.998	10.0	306	1.23	–	389
trans-ε-Viniferin	−66727[c]	66919[c]	0.997[c]	21.5	322	2.0	+	455
Flavanols								
(+)-Catechin	−214	11831	0.999	4.2	278	0.36	–	289
(−)-Epicatechin	−4836	20984	0.999	6.0	278	0.46	–	289
(−)-Epigallocatechin	*	*	*	*	*	0.31	–	305
(−)-Epicatechin gallate	*	*	*	*	*	0.65	–	441
(−)-Epigallocatechin gallate	*	*	*	*	*	0.35	–	457
Procyanidin B1	*	*	*	*	*	0.29	–	577
Flavonols								
Quercetin	−134573[c]	87227[c]	0.996[c]	22.1	371	2.15	+	303
Quercetin-3-*O*-glucoside	*	*	*	*	*	1.30	–	463
Myricetin	−119958[c]	89030[c]	0.998[c]	16.6	374	1.57	–	317
Kaempferol	−182808[c]	123799[c]	0.998[c]	26.2	365	2.65	+	287
Kaempferol-3-*O*-glucoside	*	*	*	*	*	1.69	–	447
Rutin	−9248	28334	0.998	13.4	354	1.32	–	609
Cinnamic acid derived								
trans-Cinnamic acid	27071[c]	254231[c]	0.998[c]	22.8	276	2.20	–	147
p-Coumaric acid	−24165	208546	0.998	10.8	309	0.80	+	165
trans-Ferulic acid	−14742	115639	0.997	11.0	322	1.02	+	195
Flavanonol								
Taxifolin	−23528	103730	0.998	10.5	288	0.74	+	305

[a] Linear equation: intercept (a), slope (b) and correlation factor (*r*).
[b] Equal to molecular weight in which electrospray mode (ESI^+ or ESI^-).
[c] Using the linearity results of a previously validated method (Silva et al., 2016).
* Linearity was not analyzed due to co-elution.

statistics. The different R_T and λ_{max} values allowed the selective determination of each analyte. These results are in accordance with FDA (US Food) (2001) requirements for analytical method validation.

However, when the HPLC-DAD method was tested using direct injection of a fortified red wine sample (CS1), only six BPs were detected: *trans*-resveratrol, myricetin, *trans*-ε-viniferin, quercetin, *trans*-cinnamic acid, and kaempferol (Silva et al., 2016). Fig. 2B illustrates the chromatogram for pure CS1 wine, in which these BPs were found, except for *trans*-cinnamic acid and *trans*-ε-viniferin. Despite the potential of the HPLC-DAD method, the chemical complexity of red wines limits the analysis to a small number of BP, owing to several co-elutions with undesirable compounds. To have an acceptable chromatographic separation of more BP analytes, longer times of analysis and larger amounts of solvents would be required, which is against the green chemistry principles. Therefore, we conducted a study to remove the main interferences during the LC analysis of BP.

Substantial interferences occur owing to anthocyanin pigments, causing absorption both in the visible region and the UV region, which is the range where BP should be detected. The UV/Vis spectra shown in Fig. 2C are consistent with those of red wine pigments such as malvidin 3-*O*-glucoside, an anthocyanin compound. The confirmation of such a wide region of co-elution is observed in the contour view graph of the CS1 sample directly injected in the HPLC-DAD instrument (Fig. 3A).

In these colored figures, the blue regions mean zero absorption at the corresponding retention time and wavelength. The change from green to red represents increasing peak concentrations. It was possible to confirm that, from the beginning of the chromatogram up to 15 min of elution, the analytes and some interferences elute simultaneously. In this range of retention time, high absorption intensities were observed at visible region wavelengths (400–550 nm), which are consistent with the absorption signals of anthocyanins. Peak purity determination also confirmed this, since compounds that eluted before 15 min had poor peak purity values (<95%).

Therefore, it is possible to conclude that anthocyanins are undesirable components in HPLC-DAD analysis of several BPs in red wines. Several organic red wine constituents interfere in the BP analysis, because such compounds also elute and are detected by the HPLC-DAD method within the same time scale (He & Giusti, 2011).

3.2. Choice of SPE adsorbent

Here, the main objective was to develop a method to analyze the maximum number of BPs in the same chromatographic run. An attempt was also made to determine whether the acceptable selectivity of each analyte can be obtained. The above results show that direct injection was not considered any further, and a series of SPE experiments aimed at reducing anthocyanin interferences while simultaneously quantifying various BP were carried out. Direct injection, as already proven in Section 3.1, and the C18 cartridge were not able to completely solve the selectivity problem regarding the desired analytes (Fig. 3A and B, respectively). An overlap of the UV/Vis spectra of the analytes with those of anthocyanins is observed, which demonstrates that they have not been successfully separated. The inefficiency of C18 SPE has been reported (Silva et al., 2011; Villiers et al., 2004). Rebelo, Sousa, Valentão, Rego, and Andrade (2014) employed C18 for the determination anthocyanins and some acids in wines; their conclusions agree with our on this class of compounds and the C18 cartridge. Other proposed methods have led to low analyte yields, like the use of styrene-divinylbenzene adsorbents for compounds such as quercetin and myricetin (Villiers et al., 2004).

In the present method, a different mechanism was envisioned, *i.e.* the retention of cationic anthocyanins, while the other neutral

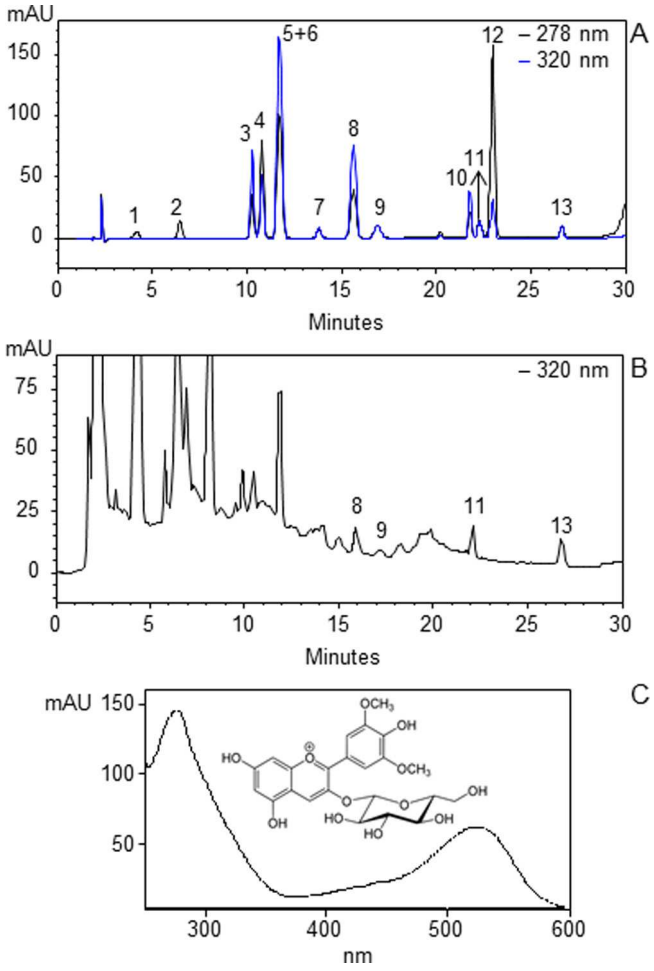

Fig. 2. HPLC-DAD method (conditions according to Section 2.4) for BP analysis: A) chromatograms at 278 and 320 nm, with 10 mg/L of each BP standard, except catechin and epicatechin (5 mg/L); B) chromatogram at 320 nm for Cabernet Sauvignon red wine (CS1) before solid phase extraction (direct injection); C) UV/Vis spectrum and chemical structure of malvidine-3-*O*-glucoside. Peak identification: (+)-catechin (1), (-)-epicatechin (2), *trans*-piceid (3), taxifolin (4), *trans*-ferulic acid (5), *p*-coumaric acid (6), rutin (7), *trans*-resveratrol (8), myricetin (9), *trans*-ε-viniferin (10), quercetin (11), *trans*-cinnamic acid (12), and kaempferol (13).

phenols elute in an acidic medium. After treatment with the SCX cartridge, the wine was free from anthocyanins. Fig. 3C and E illustrates the purified CS1 wine sample after passing through an SCX cartridge (SCXa test). Its contour graph presents a clean area in the visible range, showing that anthocyanins have not been detected. Remarkably, the compounds of interest, *i.e.*, BP, could still be detected after the SCX cartridge treatment, as shown for the fortified red wine sample (Fig. 3D).

3.3. Refinement of the SPE procedure

Once the anthocyanins have been removed from the samples, BP recovery could be further enhanced. Considering this, some variations of the SCXa test were conducted to optimize the corresponding recoveries. The organic solvents were varied and different volumes of the CS1-fortified sample were also used in these tests (Supplementary Table 1).

A comparison between the obtained recoveries for the six tested purification methods aimed at BP analysis in red wines is presented in Table 2. The analytes have been ordered according to their R_T values, which are inversely proportional to the polarity of the analytes along the chromatogram.

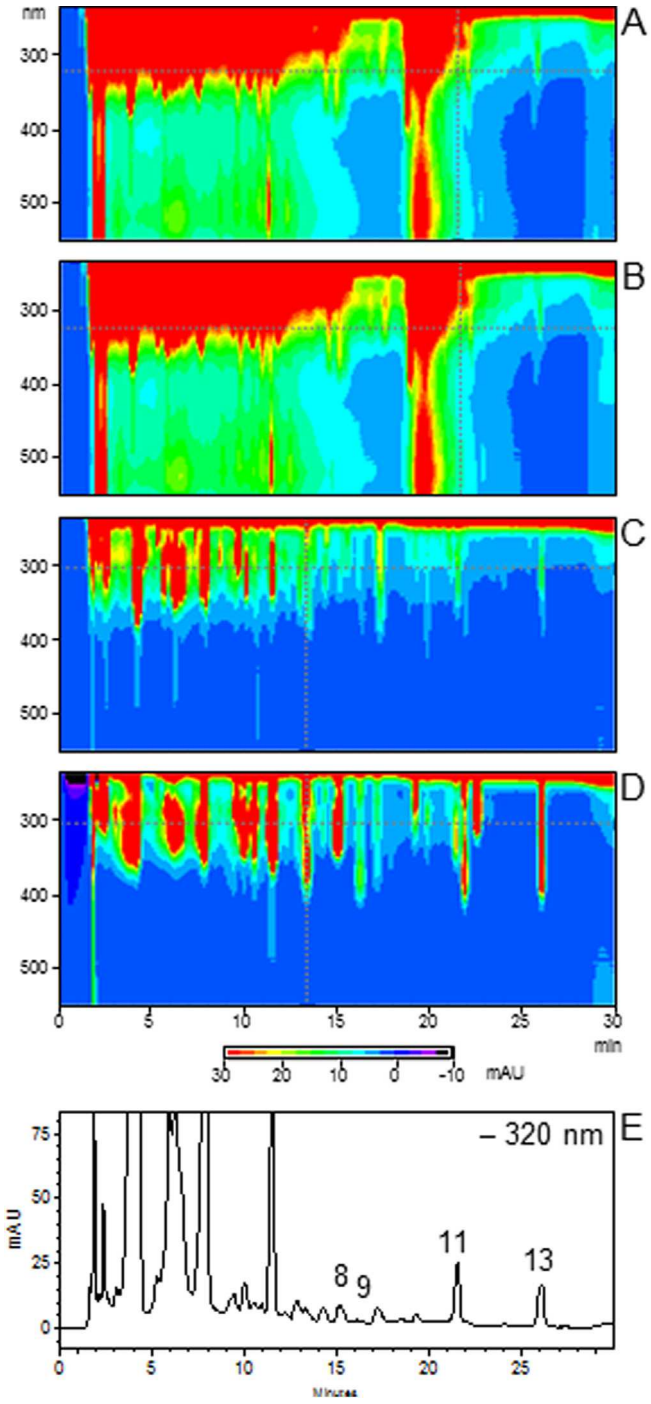

Fig. 3. HPLC-DAD contour graph of the Cabernet Sauvignon wine sample (CS1): A) original sample before solid phase extraction (SPE); B) original sample after SPE through an octadecylsilane (C18) cartridge; C) original sample after SPE by cation exchange (SCX), SCXa test; D) fortified sample, after SPE by strong cation exchange (SCX), SCXa test. E) HPLC-DAD chromatogram at 320 nm for Cabernet Sauvignon red wine (CS1) after solid phase extraction by strong cation exchange (SCX), SCXa test. HPLC-DAD conditions were conducted according to Section 2.4. The SCXa test (methanol solvent) is described in Supplementary Table 1.

Although direct injection provided higher recoveries for resveratrol, myricetin, viniferin, quercetin, cinnamic acid, and kaempferol, its lack of selectivity did not allow the quantification of all the tested analytes.

Sample preparation with the C18 cartridge did not remove the anthocyanins and thus reduced the recovery values for the same

Table 2
Recoveries from sample preparation tests and the SCXd test in real samples of red wine. HPLC-DAD conditions according to Section 2.4.

Analyte	Recovery in sample preparation tests[a]						Results with SCXd			
	Direct injection	C18	SCXa	SCXb	SCXc	SCXd	CS1	CS2	Merlot	Syrah
trans-Piceid	NS	NS	66.8 ± 4.3	38.4 ± 3.9	75.5 ± 0.2	95.3 ± 2.0	ND	ND	4.6 ± 3.7	ND
Taxifolin	NS	NS	69.5 ± 6.9	NS	91.8 ± 2.4	89.4 ± 1.8	ND	ND	ND	ND
trans-Resveratrol	94.1 ± 5.0	76.1 ± 2.7	35.2 ± 9.3	92.3 ± 0.5	91.6 ± 2.6	90.4 ± 5.3	1.7 ± 3.6	1.3 ± 1.1	<LQ	ND
Myricetin	95.0 ± 4.3	104.2 ± 3.1	10.1 ± 23.7	6.5 ± 6.7	21.0 ± 0.2	87.2 ± 4.0	3.1 ± 3.7	ND	<LQ	1.9 ± 3.1
trans-ε-Viniferin	89.2 ± 4.0	75.1 ± 2.2	14.0 ± 19.6	60.4 ± 8.1	69.0 ± 0.0	86.6 ± 4.3	<LQ	4.6 ± 0.9	1.1 ± 4.9	1.4 ± 0.3
Quercetin	98.6 ± 4.7	80.2 ± 1.9	18.9 ± 12.4	23.0 ± 0.1	35.0 ± 1.1	90.1 ± 4.1	3.6 ± 4.3	3.5 ± 4.9	<LQ	ND
trans-Cinnamic acid	94.7 ± 4.4	72.5 ± 3.5	87.4 ± 3.4	86.7 ± 0.7	91.8 ± 0.2	101.2 ± 1.3	ND	ND	ND	ND
Kaempferol	93.0 ± 4.7	88.3 ± 3.5	24.6 ± 5.6	60.4 ± 0.6	56.1 ± 2.6	99.5 ± 5.3	2.5 ± 3.2	3.6 ± 2.3	<LQ	ND

NS: Not selective, with peak purity below 95%.
ND: Not detectable, over the limit of detection.
LQ: Limit of quantification.
CS: Cabernet Sauvignon sample.
[a] Mean recovery (n = 3) of a fortified wine (10 mg/L of each analyte standard) and its corresponding RSD%.

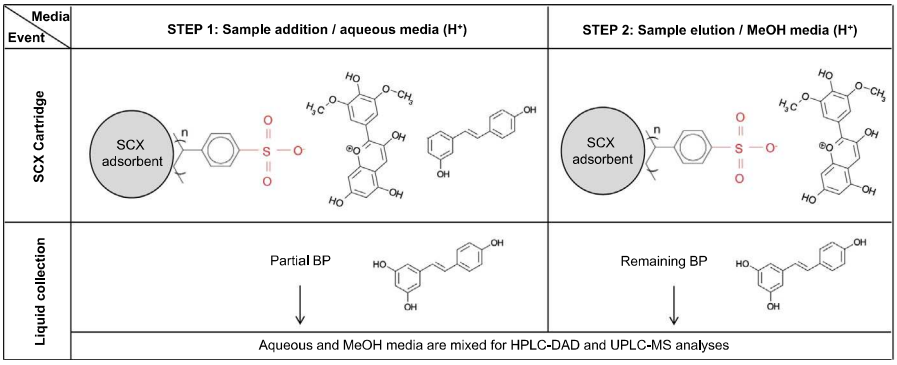

Fig. 4. Elution sequence of the purification process of bioactive phenols (BPs) in a red wine sample by solid phase extraction through a strong cation exchange (SCX) cartridge with methanol solvent (SCXd test). Step 1: preparation sequence. Step 2: elution sequence. Representative chemical structures: anthocyanin (positively charged in acid media) and other bioactive phenols (with a neutral charge in acid media).

six BPs analyzed by direct injection (Silva et al., 2016). The tests with the SCX cartridges provided higher recoveries in the ascending order: SCXa, SCXb, SCXc, and SCXd. In particular, SCXd showed recoveries from 86.6% to 101.2% (the lowest value corresponds to *trans*-ε-viniferin and the highest one to *trans*-cinnamic acid), accordance with validation requirements (FDA, 2001). All the test results show that SCXd was able to quantify more BPs, as observed on the radar graph (Supplementary Fig. 1). Some differences were found by changing the nature of the eluent and the sequence, since SCX also enables the use of other mechanisms of retention such as π-π bonding and hydrophobic interactions (Moldoveanu & David, 2002), owing to different polarities of the analytes. Kaempferol, *trans*-ε-viniferin, and quercetin, for example, are less polar than the studied compounds and present therefore a stronger interaction with the sorbent.

The SCXa and SCXb tests were used to concentrate the analytes in the elution sequence, since, sometimes, they may be present at low concentrations in red wine samples. However, the most polar analytes such as piceid, viniferin, resveratrol, and myricetin were partially lost through the preparation sequence. Even after this loss, it is possible that the alcohol content in wine (around 12% v/v) was enough to elute some of them before eluent addition. Hence, further attempts (SCXc and SCXd) were made by joining the fractions of wine and eluent, thereby diluting the sample twice.

The SCXd test, with an eluent composed of acidic MeOH, proved to be the most appropriate to elute the analytes with no interference between the compounds. Such purification method allowed high recoveries of the quantifiable compounds. Furthermore, the SCXd method presented acceptable recoveries in comparison with those achieved by the strong anion exchange method, the former having the advantages of being simpler requiring lower solvent consumption (Figueiredo-González et al., 2014; Guillén et al., 1996; Guillén et al., 1997).

As the anthocyanin interferences are adsorbed on the cartridge, the SCXd treatment eliminates the need for a final washing step with an alkaline solution. After sample elution, the cartridge may be directly discarded, without the need for any further addition of an organic solvent, in agreement with the green chemistry principle of waste reduction (Tobiszewski & Namiesnik, 2012).

3.4. Additional analytes by UPLC-MS

For a better exploration of the SCX potential to analyze a wide number of BPs in red wines, a UPLC-MS method was applied. Determining the co-eluted BP by modification of the HPLC-DAD method (described in Section 2.4) would probably imply longer analysis times and larger solvent quantities. UPLC-MS, in principle, presents no co-elution problems, since its detector is more selective toward each analyte. Despite this, sample purification is also desirable for this instrument, even if only for the purpose of column preservation.

After passing a fortified CS1 sample through SCX (test SCXd), 19 BPs were identified by UPLC-MS. Each R_T, the ESI mode with the highest sensibility and the corresponding *m*/*z* is presented in Table 1. The *m*/*z* detected for each BP was similar to those previously established (Vrhovsek et al., 2012). These results show that the SCX treatment can also be used to analyze other BPs in red wines.

Moreover, anthocyanins were not detected after the fortified sample passed through the SCX cartridge, as positively charged

molecules are retained on the SCX column surface (Fig. 4). On the contrary, SCX allows the elution of BPs, which are neutral and polar to moderately polar compounds. The pKa of analytes is higher than that of anthocyanins and they elute through the cartridge in acidic media. This difference in their interaction with the column can be explained as follows: anthocyanin molecules (Fig. 2C) are positively charged in the high acidic medium of the system, but not bioactive phenols (Fig. 1). The sorbent is a polymer bonded to benzenesulfonic acid, which is a strong cation exchanger. It retains strong cationic compounds like anthocyanins, which are protonated at very low pH values (Asenstorfer, Iland, Tate, & Jones, 2003). In contrast, the desired analytes are not retained by SCX because they are weak organic bases (*e.g.* resveratrol, $pka_1 = 8.99$, $pka_2 = 9.63$, $pka_3 = 10.64$) or weak organic acids (*e.g.* coumaric acid, pKa = 4.0) (Chemicalize, 2016).

3.5. Quantification of real samples of red wines

Typical varieties of red wines were employed to test the SCXd purification procedure. The results for the sample used in the previous tests (CS1), another Cabernet Sauvignon sample (CS2), Syrah, and Merlot samples are presented in Table 2. Peaks of the sample analytes were compared to the standard profiles by means of their purity (>95%), retention time (RSD < 5%) and UV/Vis spectra.

BPs profile distinguished the CS1 and CS2 samples, which were from the same region and variety (Cabernet Sauvignon), but from different wineries. *trans*-resveratrol, *trans*-ε-viniferin, quercetin, and kaempferol were found in the CS1, CS2 and Merlot samples, with higher values obtained in the Cabernet Sauvignon samples than in the Merlot one. In contrast, the Merlot sample contains 4.63 mg/L of piceid, which was not detected in the CS1, CS2, and Syrah samples. The Cabernet Sauvignon and Merlot samples contained higher BP values than the Syrah sample. Taxifolin and *trans*-cinnamic acid were not found in any of the analyzed samples.

4. Conclusions

Our results prove that anthocyanins interact strongly with the SCX sorbent in acidic media, being removed from the red wine samples. When evaluated by the HPLC-DAD method, the SCX purification treatment increases the number of quantifiable analytes, compared to those with direct injection and the C18 cartridge. UPLC-MS confirmed that the SCX mechanism may be a potential tool to separate BP analytes from anthocyanins, a major source of interference in red wine analysis. The SCX purification procedure conferred selectivity to the analytical method as the interference of anthocyanins was eliminated. Surprisingly, there are no previous reports regarding this specific purpose.

By using BPs as markers of the nutraceutical grade of wines, it is possible to study and establish the best conditions for field management, and the potential regions for each grape variety and winemaking technologies. This SCX method can also be adapted for the treatment of other matrices such as grapes related products or several other fruits. Furthermore, this is a promising SPE mechanism for the analysis of other organic compounds with chemical characteristics similar to those of BP (*e.g.* amines or organic acids) or for other analytical methods (*e.g.* spectroscopy).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.01.087.

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