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Analytical Methods

Development of a high-performance liquid chromatography method based on a core–shell column approach for the rapid determination of multiclass polyphenols in grape pomaces

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ABSTRACT

A rapid and economically affordable reverse-phase chromatographic approach based on a core–shell column with high-performance liquid chromatography multi-wavelength detector (HPLC-MWD) is proposed for the quantification and quality control of multiclass polyphenols (PPs). The separation of 20 relevant polyphenols from grape pomace extracts (GPEs) was achieved in less than 12 min by using a Kinetex C_{18} column (3.0 mm × 100 mm, 2.6 μm) with a gradient system of ultrapure water (0.1% formic acid) and acetonitrile, a temperature of 35 °C and a flow rate of 0.8 mL min^{-1}. The maximum backpressure reached was 327 bar, meaning the developed method is adequate for standard HPLC instruments. The applicability of the method was demonstrated by the determination of PPs in GPEs of different red grape varieties. Cabernet Sauvignon GPE showed the highest content of studied PPs (9804.2 μg g^{-1} GPE) followed by Bonarda GPE (7302.0 μg g^{-1} GPE). Besides the methodological development for a high throughput routine quality control of GPEs, this is the first report of PPs content for Bonarda and Aspirant Bouchet GPE, so the results add knowledge for these grape varieties cultivated in Argentina.

1. Introduction

Phenolics are secondary metabolites of plants, fruits and vegetables that contribute to several organoleptic attributes and nutritional properties (Landete, 2012; Lapornik, Prošek, & Golc Wondra, 2005). They constitute a heterogeneous family including phenolic acids, flavonoids, tannins, stilbenes, coumarins, lignans and phenylethanol analogs (Garrido & Borges, 2013). Polyphenols have been reported that possess health-promoting effects in different biological systems as antioxidant, anti-inflammatory, anti-carcinogenic, anti-mutagenic and anti-proliferative activities (González-Vallinas, González-Castejón, Rodríguez-Casado, & Ramírez de Molina, 2013), highlighting the importance of their identification and quantification.

Grape pomace (GP) is the most abundant residue of winemaking industry consisting mainly of skins and seeds that remain after fermentation and maceration during wine elaboration (Fontana, Antoniolli, & Bottini, 2013; Pinelo, Arnous, & Meyer, 2006). This by-product contains relatively high levels of polyphenols due to an incomplete extraction during winemaking process (Fontana et al., 2013). Therefore GP could be an alternative source of natural antioxidants to minimize the use of synthetics that are widely used in the food industry. As well, the exploitation and valorization of industrial byproducts are a strategy used by modern industries to diminish the environmental impact of their residues (Fontana et al., 2013). Therefore, most attention has been paid on the recovery of bioactive phenolics from grape byproducts of the winemaking industry and its further use in pharmaceutical and food industries.

The chemical characteristics of GP extracts (GPEs) are related to the content of phytochemicals with bioactive properties (Fontana et al., 2013), and so it is of utmost significance to determine their composition. These data may provide valuable information for the characterization of samples and likewise increase the value of the product. In this sense, highly efficient analytical methodologies for identification and quantification of compounds are necessary for these objectives to succeed.

Chromatographic techniques, especially high-performance liquid chromatography (HPLC) coupled to different detectors such as UV–Vis, multi-wavelength (MWD) and mass spectrometry

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(MS) have been the choice for the analysis of polyphenols (de Rijke et al., 2006; Kalili & De Villiers, 2011; Pyrzynska & Biesaga, 2009; Valls, Millán, Martí, Borràs, & Arola, 2009). Nowadays, novel separation techniques with ultrahigh-pressure systems (UHPLC) can achieve rapid, versatile, and high-throughput separations, particularly suitable for the analysis of complex samples such as those coming from plant extracts (Ky, Lorrain, Kolbas, Crozier, & Teissedre, 2014). The technological developments in HPLC have resulted in UHPLC instruments capable of superior resolutions, but that can be expensive to the average laboratory, or difficult to adapt from known procedures (Hayes, Ahmed, Edge, & Zhang, 2014).

An alternative to improve HPLC separation efficiencies and speed without reducing particle size is the use of superficially porous particles, also named core–shell. They are typically composed of a 1.9 μm solid core enclosed by a 0.35–0.5 μm porous shell (dp = 2.6–2.7 μm), providing reduced band broadening and outstanding efficiency, while preserving sufficient particle size to allow an acceptable operation pressure (Guiochon & Gritti, 2011; Hayes et al., 2014; Jandera, Hájek, & Staňková, 2015). The shorter diffusion allows faster mass transfer, resulting in sharper and higher peaks equivalent to, or better than, sub-2 μm fully porous particles. Advantages of this technology rely on high peak efficiency without using very high-pressure HPLC instrumentation necessary for sub-2-μm fully porous particles. That is, this new technology allows UHPLC performance on regular HPLC instruments with increased reproducibility, resolution, sensitivity and flexibility along with shorter analysis times and lower solvent consumption than traditional HPLC methods.

The use of core–shell particle columns represents a promising approach for achieving high speed and high resolution analyses and its application for polyphenol determination has been reported for different matrixes (Cvetkovikj et al., 2013; Fanali, Rocco, Aturki, Mondello, & Fanali, 2012; Gómez-Caravaca, Verardo, Berardinelli, Marconi, & Caboni, 2014; Jandera et al., 2015; Manns & Mansfield, 2012). Considering the complexity of GPE, which includes high quantity of target analytes of different chemical nature and levels of concentration, PPs analysis represents a noteworthy challenge in terms of achieving an efficient separation in shorter times. In this way, the objective of this work was to develop a rapid, simple, and cost-effective HPLC analytical method using a core–shell chromatography approach coupled to multiple wavelength detection (MWD) for the simultaneous determination of 20 polyphenols representatives of different chemical classes (phenolic acids, flavanols, flavonols, stilbenes and phenylethanol analogs) in GPEs. The optimized method has been applied for the characterization and quantification of the major polyphenolic constituents of GPE coming from different grape varieties cultivated in Mendoza, Argentina, to expand the knowledge about polyphenols composition of GPE and increase the value of the product.

2. Experimental

2.1. Standards, solvents and sorbents

Standards of gallic acid (99%), 3-hydroxytyrosol (⩾99.5%), (−)-gallocatechin (⩾98%), (−)-gallocatechin gallate (⩾99%), caftaric acid (⩾97%), (−)-epigallocatechin (⩾95%), (+)-catechin (⩾99%), (−)-epicatechin (⩾95%), caffeic acid (99%), syringic acid (⩾95%), coumaric acid (99%), ferulic acid (⩾99%), polydatin (⩾95%), piceatannol (99%), *trans*-resveratrol (⩾99%), quercetin hydrate (95%), cinnamic acid (99%), quercetin 3-β-D-glucoside (⩾90%), kaempferol-3-glucoside (⩾99%) were purchased from Sigma–Aldrich. The standard of 2-(4-hydroxyphenyl) ethanol (tyrosol) (⩾99.5%) was obtained from Fluka (Buchs, Switzerland). Stock solutions of the above compounds were prepared in methanol at concentration levels of 1000 μg mL^{-1}. Further dilutions were prepared monthly in methanol and stored in dark-glass bottles at −20 °C. Calibration standards used during optimization of HPLC-MWD conditions were dissolved in ultrapure water (0.1% formic acid; FA)/Acetonitrile (MeCN) (95:5).

HPLC-grade MeCN and FA were acquired from Mallinckrodt Baker (Inc. Pillispsburg, NJ, USA). Analytical grade sorbents (50 μm particle size) for dispersive solid phase extraction (d-SPE), including primary-secondary amine (PSA) and octadecylsilane (C_{18}) were both obtained from Waters (Milford, MA, USA). Reagent grade NaCl, anhydrous $MgSO_4$ and anhydrous $CaCl_2$ were purchased from Sigma–Aldrich. Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

Table 1
Analytical performance and chromatographic characteristics of the proposed method.

	t_R (min)	λ (nm)	Width 50% (min)	Linear range (μg mL^{-1})	Calibration curve	r^2	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	Intra-day[a]		Inter-day[b]	
									t_R	Peak area	t_R	Peak area
Gallic acid	1.10	280	0.035	0.5–25	$0.4452x + 0.0791$	0.9997	0.08	0.26	0.01	0.27	0.06	1.11
(−)-Gallocatechin	2.01	254	0.051	0.5–10	$0.07x + 0.0139$	0.9996	0.10	0.33	0.10	1.09	0.21	1.10
OH-tyrosol	1.89	280	0.043	0.5–25	$0.108x + 0.0055$	0.9995	0.09	0.30	0.08	1.21	0.12	0.95
Caftaric acid	2.63	320	0.061	1–25	$0.2521x - 0.0869$	0.9980	0.15	0.5	0.09	1.70	0.18	1.99
Tyrosol	3.29	280	0.059	0.5–25	$0.0856x + 0.0081$	0.9995	0.11	0.36	0.07	1.61	0.09	1.49
(−)-Epigallocatechin	4.36	254	0.049	1–25	$0.0188x + 0.0032$	0.9998	0.27	0.90	0.08	1.05	0.11	1.87
(+)-Catechin	4.72	280	0.048	1–100	$0.0966x - 0.2056$	0.9951	0.26	0.86	0.09	1.08	0.09	1.63
Caffeic acid	5.21	320	0.047	0.5–25	$0.4948x + 0.0347$	0.9996	0.12	0.40	0.07	1.99	0.08	1.92
Syringic acid	5.67	280	0.043	0.5–25	$0.2687x + 0.024$	0.9995	0.10	0.33	0.05	1.18	0.05	1.27
(−)-Epicatechin	5.98	280	0.039	0.5–50	$0.089x + 0.0116$	0.9998	0.13	0.43	0.03	1.04	0.05	1.67
p-Coumaric acid	6.76	320	0.047	0.5–25	$0.6249x + 0.0614$	0.9995	0.09	0.30	0.02	0.99	0.05	1.19
(−)-Gallocatechin gallate	6.92	280	0.042	0.5–10	$0.7331x + 0.1752$	0.9981	0.12	0.40	0.02	2.81	0.04	2.91
Ferulic acid	7.49	320	0.045	0.5–25	$0.4868x + 0.0318$	0.9996	0.07	0.23	0.03	1.02	0.03	1.53
Polydatin	7.79	320	0.038	0.5–25	$0.3826x + 0.0197$	0.9995	0.07	0.23	0.02	0.78	0.02	1.68
Piceatannol	8.35	320	0.045	0.5–10	$0.3536x + 0.0961$	0.9980	0.11	0.36	0.03	1.42	0.03	3.61
Quercetin-3-glucoside	8.20	370	0.037	0.5–10	$0.2039x - 0.0828$	0.9947	0.12	0.40	0.02	0.55	0.02	0.99
Kaempferol-3-glucoside	8.90	370	0.036	0.5–10	$0.2186x - 0.0782$	0.9954	0.10	0.33	0.02	0.96	0.02	2.06
Trans-resveratrol	9.87	320	0.044	0.5–25	$0.9252x + 0.0454$	0.9990	0.08	0.26	0.01	1.42	0.04	2.44
Cinnamic acid	10.37	280	0.051	0.5–25	$0.7578x + 0.1003$	0.9996	0.14	0.46	0.02	0.78	0.02	1.24
Quercetin	10.85	370	0.049	0.5–25	$0.2056x - 0.2453$	0.9910	0.33	1.10	0.03	2.56	0.03	4.17

[a] $n = 5$ replicate injections in the same day (2.5 μg mL^{-1}).
[b] $n = 15$ injections in 3 consecutive days (2.5 μg mL^{-1}).

2.2. Samples and sample preparation

This study was performed with GP obtained from three different *Vitis vinifera* L. cultivars: Cabernet Sauvignon, Bonarda and Aspirant Bouchet, provided by Catena Institute of Wine from different vineyards located in Mendoza, Argentina, and harvested in 2013. The vinification procedure was conducted with daily pumping and contact of the skins and seeds with the juice for 11 days. After that, must was pressed, the fresh GP samples collected, placed in ice cooled boxes for transportation to the laboratory, and stored at −20 °C until processing.

The recovery of polyphenols from GP was performed by solid–liquid extraction. Conditions were chosen from literature and some adjustments were performed (Amendola, De Faveri, & Spigno, 2010; Bucic-Kojic, Planinic, Tomas, Jakobek, & Šeruga, 2009; Spigno, Tramelli, & De Faveri, 2007; Vatai, Skerget, & Knez, 2009). Eighty grams of fresh GP samples were ground in a laboratory mixer with an aliquot of the extraction solvent (ethanol:water, 50:50 v/v) at a 5:1 solvent-to-sample ratio. The extraction was carried out during 2 h under continuous stirring at 60 °C. The liquid was filtered through a filter paper and concentrated in a rotary evaporator at 40 °C. The concentrated extracts were freeze-dried for 96 h at 0.12 bar and −45 °C (Free Zone 2.5, Lab Conco, Missouri, USA). Freeze-dried extracts were placed in sealed tubes and kept at −20 °C in dry atmosphere and darkness prior analysis.

PPs were extracted as previously reported with some modifications according to the analyzed sample (Fontana & Bottini, 2014). Briefly, 50 mg of freeze-dried extract were dissolved in water, made up to 5 mL and extracted with 2.5 mL acidified (1% FA) MeCN. For phase separation, 1.5 g NaCl and 4 g anhydrous $MgSO_4$ were added, shaken 1 min and centrifuged 10 min at 3000 rpm. Then, 1 mL aliquot of the upper MeCN phase was transferred to a 2 mL d-SPE clean-up tube containing 150 mg anhydrous $CaCl_2$, 50 mg PSA and 50 mg C_{18}. The mixture was then vortexed 30 s and centrifuged 2 min at 12,000 rpm (8400 rcf). Finally, an aliquot of extract was evaporated to dryness; the residue was reconstituted in the initial mobile phase and analyzed by HPLC-MWD.

2.3. HPLC-MWD analysis

Target polyphenols were determined using a HPLC-MWD system (Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany). The HPLC instrument was a Dionex Ultimate 3000 consisting of vacuum degasser unit, autosampler, quaternary pump and chromatographic oven. The detector was a Dionex MWD-3000 (RS) model with an analytical flow cell operated with a data collection rate of 5 Hz, a band width of 4 nm and a response time of 1.000 s. The working wavelengths for the different families of analytes were 254 nm, 280 nm, 320 nm and 370 nm (see Table 1 for PPs identification). The Chromeleon 7.1 software was used to control all the acquisition parameters of the HPLC-MWD system and also to process the obtained data.

HPLC separations were carried out in reversed-phase Kinetex C_{18} column (3.0 mm × 100 mm, 2.6 μm) Phenomenex (Torrance, CA, USA). Ultrapure water with 0.1% FA (A) and MeCN (B) were used as mobile phases. Analytes were separated using the following gradient: 0–2.7 min, 5% B; 2.7–11 min, 30% B; 11–14 min, 95% B; 14–15.5 min, 95% B; 15.5–17 min, 5% B: 17–20, 5% B. The mobile

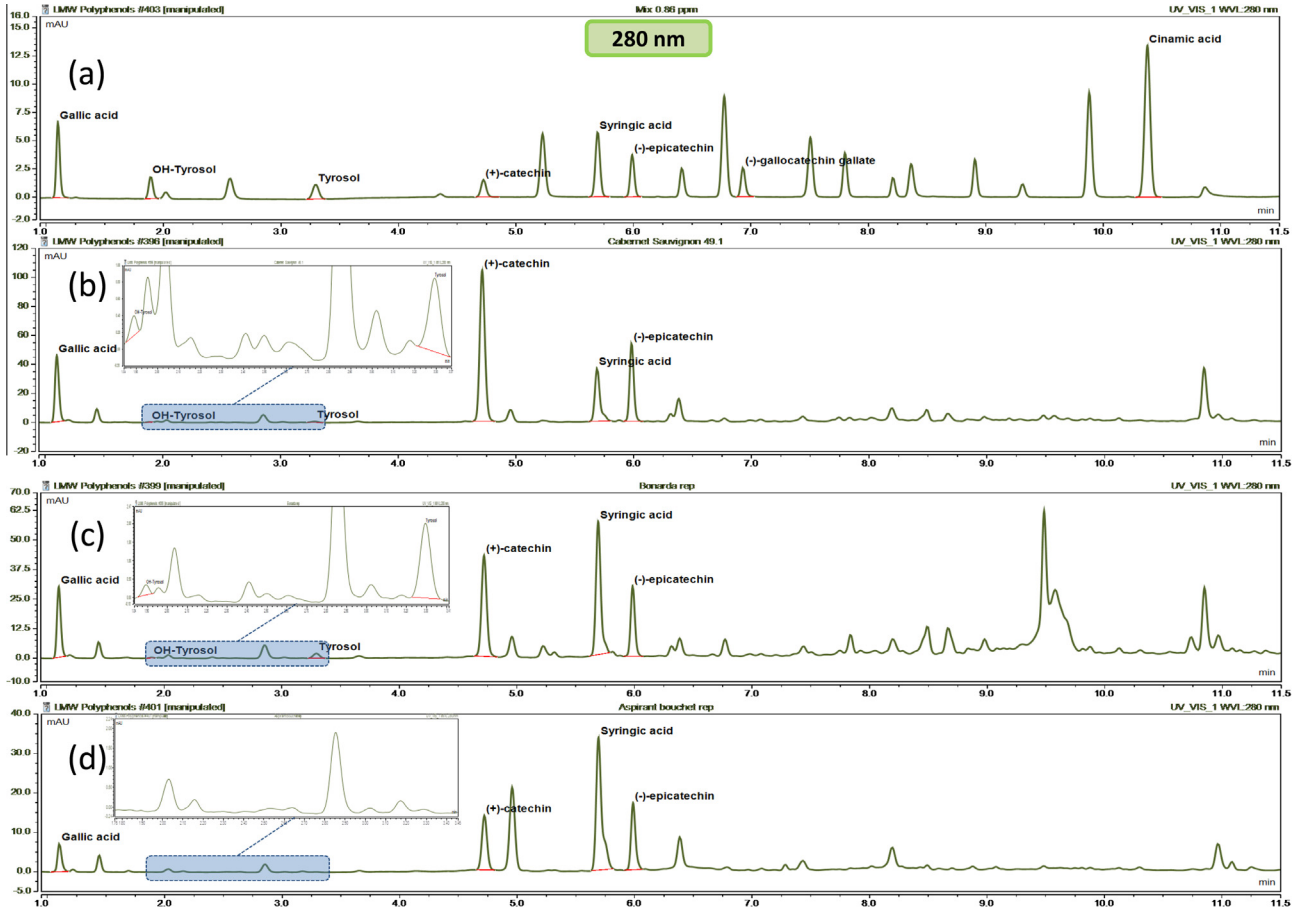

Fig. 1. HPLC-MWD extracted chromatograms recorded at 280 nm, 320 nm, 370 nm and 254 nm of (a) solvent standard at 1 μg mL^{-1} level; (b) sample of Cabernet Sauvignon GPE; (c) sample of Bonarda GPE and (d) sample of Aspirant Bouchet GPE.

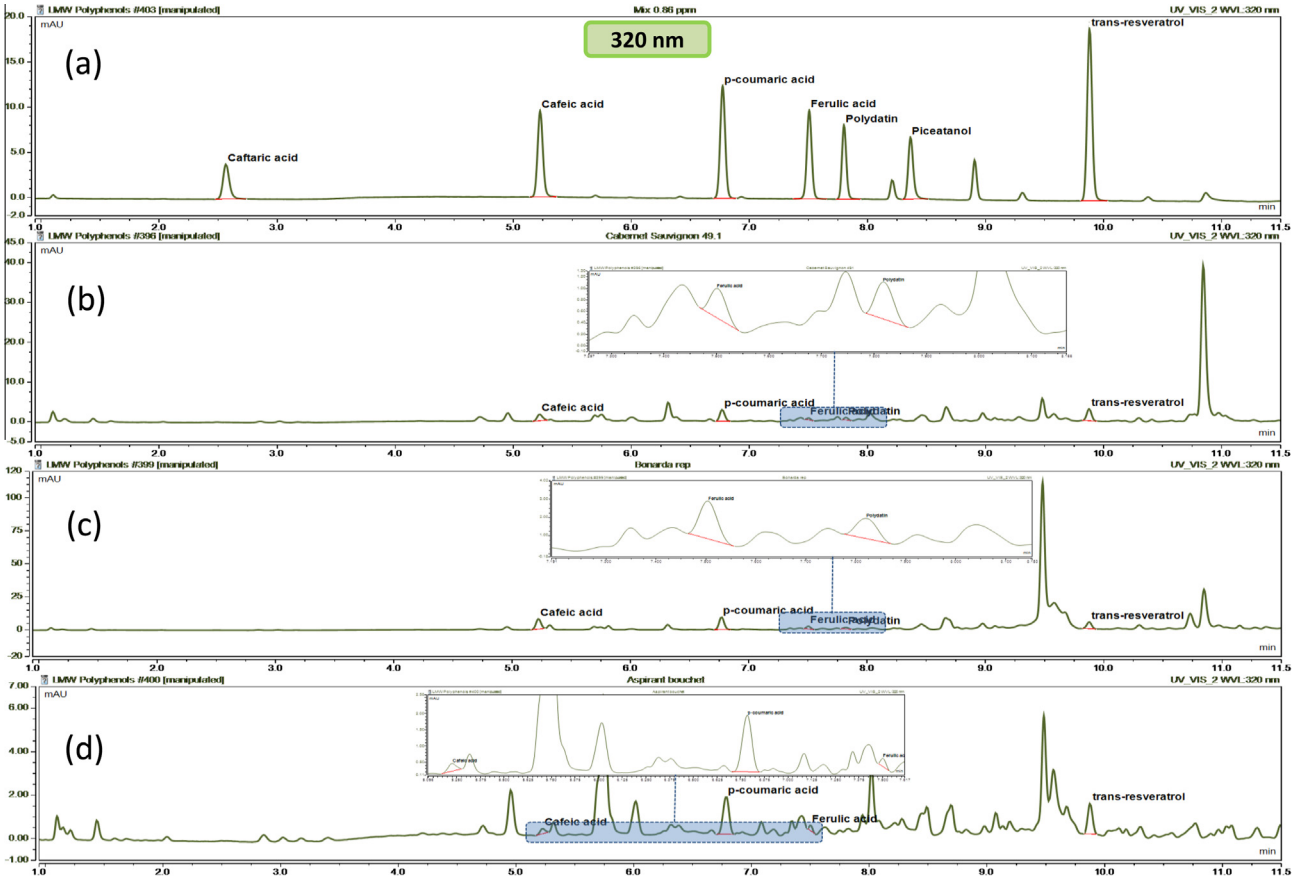

Fig. 1 (*continued*)

phase flow was 0.8 mL min^{-1}. The column temperature was 35 °C and the injection volume 5 μL. The identification and quantification of PPs in the GPE were based on the comparison of the retention times (t_R) and absorbance values of detected peaks in samples with those obtained by injection of pure standards of each analyte. Additionally, samples were spiked with known concentrations of compounds in order to verify the peak identification and the absence of interferences at the analytes t_R. Samples were quantified by using an external calibration with pure standards of each compound.

3. Results and discussion

3.1. Development of the chromatographic method

A method was developed and optimized for the separation and quantification of phenolic compounds of different families, namely phenolic acids, flavonols, flavanols, stilbenes and phenylethanol analogs by using a core–shell chromatography approach. The column used was chosen because of the special features that core–shell particles present even when used in traditional HPLC systems, including speed of analysis, good efficiency, and reduced plate heights with moderate sample loading capacity and backpressure (Ali, Al-Othman, Nagae, Gaitonde, & Dutta, 2012). The major factors affecting the separation of studied compounds were evaluated in order to achieve the best resolution of analytes in the shortest time. For this, a mixture of 20 PPs standards was used. Fig. 1a shows the chromatograms for each detection wavelength of standards mixture by applying the optimized separation conditions detailed in Section 2.3.

Separation conditions were optimized using a Kinetex C_{18} column (3.0 mm × 100 mm, 2.6 μm) and a binary mixture of water containing 0.1% FA (v/v):MeCN as elution system. The C_{18} stationary phase was selected because is the most often used to determine phenolic compounds in diverse types of matrices with excellent separation power for different families of compounds (Valls et al., 2009). As well, is a multipurpose phase which is commonly found in all laboratories working in chromatography. To carry out the optimization, a method previously described by our group, using a conventional C_{18} column was evaluated (Fontana & Bottini, 2014). This method was initially used for the separation of 10 PPs with a total run time of 32 min. By using these starting conditions with the core–shell column, the chromatographic peaks eluted too early and most of them overlapped. Thus, different modifications of flow rate, composition of solvents and column temperature were evaluated. Keeping 5% B for 2.7 min, then with a progressive increase of B (5–30%) between 2.7 and 11 min allowed better sensitivity and separation of the stilbene polydatin and ferulic acid, a critical pair during method optimization. Using these conditions, well-resolved chromatograms of standards and GPEs were obtained at a flow rate of 0.8 mL min^{-1} and column temperature of 35 °C (Fig. 1). Peak characteristics and identification are shown in Table 1. According to Fig. 1, a rapid and selective separation was achieved in less than 12 min (20 min from injection-to injection) with baseline resolution for the 20 studied analytes. This gradient effectively separated hydroxycinnamic acids, flavanols, flavonols, stilbenes and phenylethanol analogs demonstrating the separation efficiency of the proposed core–shell column approach. As well, the core–shell technology allowed narrowed peaks and excellent analytical loading for studied polyphenols.

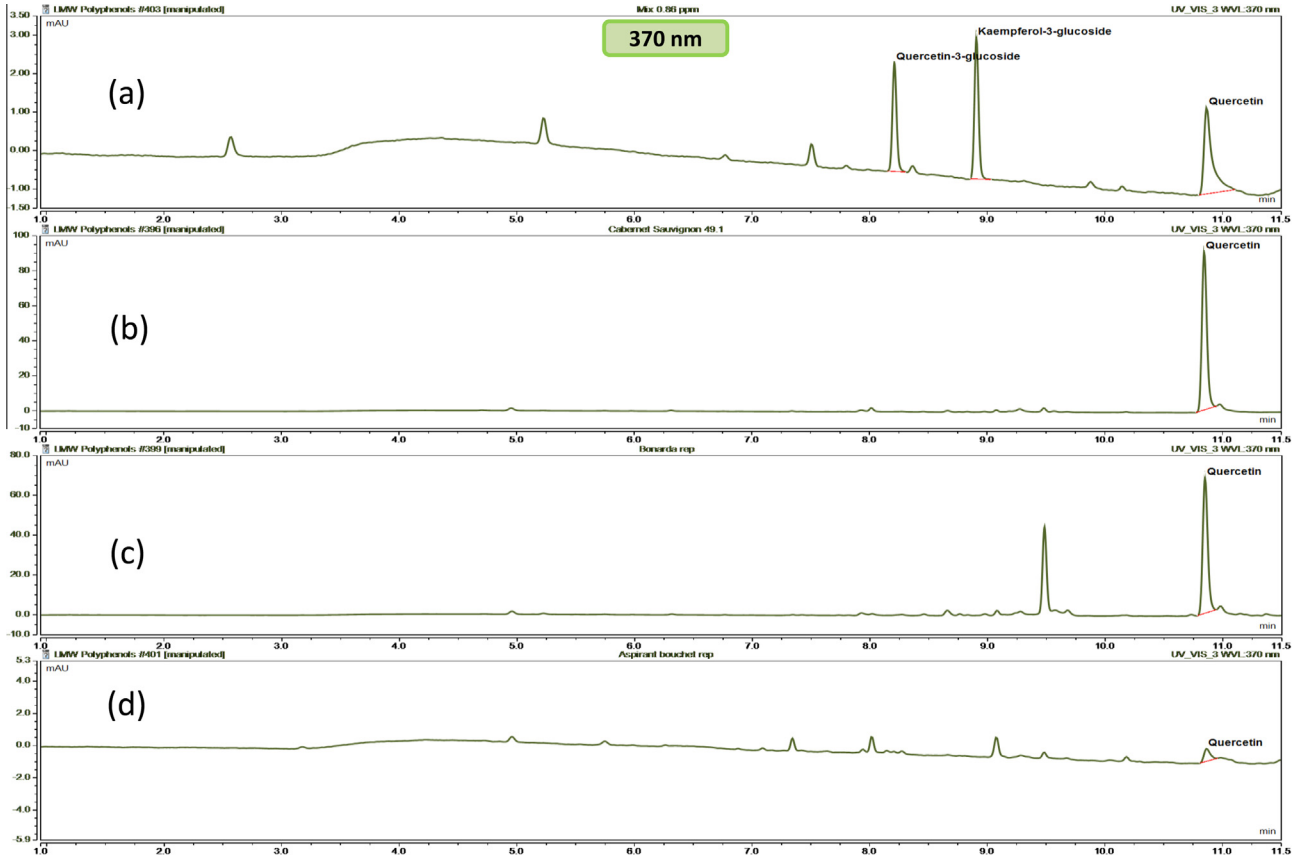

Fig. 1 (*continued*)

The proposed core–shell HPLC approach, as compared with previous works using traditional columns, showed a substantial difference in total analysis time for the determination of similar families of compounds (20 min versus ca. 50 min) (Barcia et al., 2014; Fontana & Bottini, 2014; Perestrelo et al., 2012; Ramirez-Lopez, McGlynn, Goad, & Mireles DeWitt, 2014; Restivo, Degano, Ribechini, & Colombini, 2014). The core–shell technology allows fast equilibrium that favors mass transfer, and shorter re-equilibration time in comparison with traditional columns, so increasing the sample throughput in a routine work (Hayes et al., 2014). In comparison with reports using core–shell columns for similar analytes, the proposed method achieves the separation of 20 analytes of all the families of PPs at a flow rate of 0.8 mL min^{-1} with a total run time similar to that reported by Manns and Mansfield (2012). They proposed a method for the separation of 16 PPs, but important families of phenolic compounds such as stilbenes and glucosilated flavonols were not included. As well, a flow rate of 2 mL min^{-1} was used. By comparing with UHPLC reported methods, Silva, Haesen, and Câmara (2012) achieved the separation of 15 PPs in 14 min using a C_{18} column (2.1 mm × 100 mm, 1.8 μm), with a flow rate of 0.25 mL min^{-1} and a maximum back pressure of 414 bar. The advantages of UHPLC in terms of superior resolutions, short analysis time and lower solvent consumption are undeniable. However, from an economical point of view, UHPLC systems could be too costly for most laboratories. Taking this in mind, the method proposed here with a core–shell column is able to achieve comparable and cost-effective results on an average laboratory. In this sense, it could be applied for quantification of a considerable number of PPs in GPEs and other matrixes, noticeably plant extracts, without spending much time and expenses.

3.2. HPLC method validation

The analytical figures of merit for the optimized method are summarized in Table 1. Quantification was made according to the linear calibration curves of standard compounds. The calibration curves were constructed with six levels of concentration in triplicate. Linear ranges between 0.5 and 10 or 25 μg mL^{-1} as maximum level were obtained, with the exception of some analytes as is detailed in Table 1. All the curves gave r^2 values higher than 0.9910 in the studied range of concentration. The LODs of the analytes, calculated as three times the signal-to-noise ratio ($S/N = 3$), were ranged between 0.07 for ferulic acid and polydatin to 0.33 μg mL^{-1} for quercetin. The achieved LODs showed that the proposed method shows a suitable sensitivity according to the polyphenol levels commonly found in GPE. The precision was evaluated through inter-day (reproducibility) and intra-day (repeatability) studies, calculating the relative standard deviation using the retention times and peak area of each polyphenol (Table 1). For reproducibility, the RSD values were under 0.10% (for retention time, (−)-gallocatechin) and 2.81% (for peak area, (−)-gallocatechin gallate). The inter-day precision was assessed by analyzing a standards solution (2.5 μg mL^{-1}) in 5 replicated injections during three consecutive days. The calculated RSDs for the studied polyphenols were lower than 0.21% ((−)-gallocatechin) and 4.2% (Quercetin) for retention times and peak area,

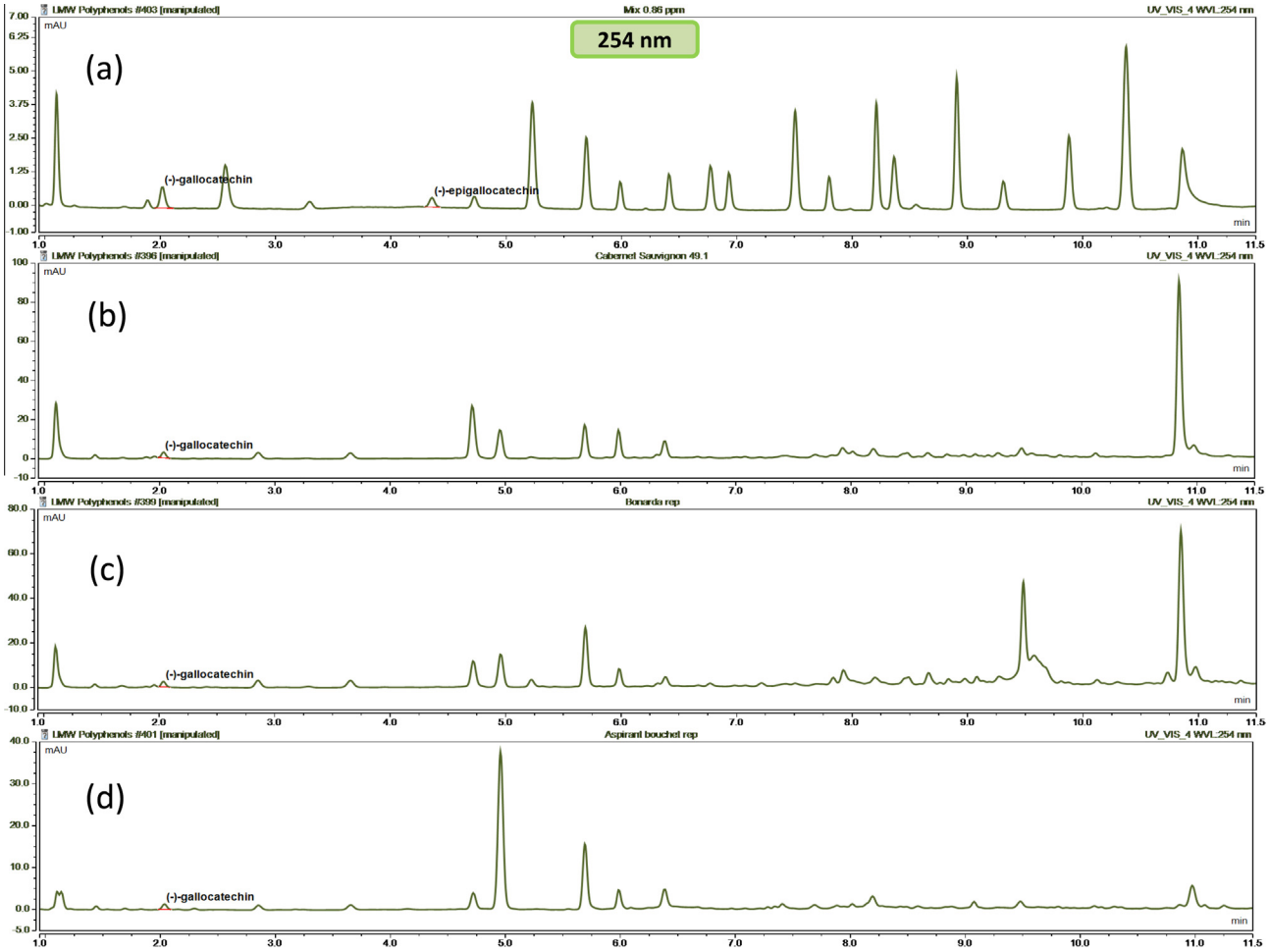

Fig. 1 (*continued*)

respectively. Table 1 summarizes the intra- and inter-day precision data for retention times and peak areas.

The selectivity of the method for the quantification of PPs in GPEs was evaluated by the comparison of t_R achieved by analyzing a standard solution of polyphenols and a GPE extract (with and without spiking analytes). As can be observed from Fig. 1, the GPE matrixes do not affected the t_R and peaks were properly identified and quantified without observing interferences at their t_R.

The absolute recoveries (R, %) of the proposed method were calculated as the difference between the concentrations measured for extracts from spiked (C_s) and non-spiked aliquots (C_b) of GPE divided by the theoretical concentration (C_t) added to the sample, and multiplied by 100,

$$R\ \% = \left[\frac{C_s - C_b}{C_t}\right] \times 100$$

where C_s and C_b were established against calibration curves obtained by external calibration. The recoveries, considered as an estimation of the accuracy, were assessed using Bonarda GPE spiked at two different concentration levels (2.5 and 25 $\mu g\ g^{-1}$). The obtained results are summarized in Table 2. In all cases, spiked and non-spiked aliquots were processed in triplicate and analytes concentrations were determined from calibration curves. The obtained values ranged between 69.2% ((−)-gallocatechin) and 124.3% ((+)-catechin) with associated standard deviations between 5.5% and 12.1%.

3.3. Application of developed method for quantification of PPs in GPE samples

The PPs composition of GPEs from the three red grape varieties investigated is described in a detailed form in Table 3. All the extracts were analyzed by using the developed HPLC-MWD method to identify and quantify the PPs content. The analytes were successfully separated and identified by comparing their retention times with pure standards. Good peak shape and resolution were achieved for all the compounds with low interference from GPE matrix (Fig. 1).

In this study, a total of 13 polyphenols were detected and quantified in GPE of Bonarda, Cabernet Sauvignon and Aspirant Bouchet winemaking by-products. The quantified PPs were gallic, syringic, caffeic, p-coumaric and ferulic acids, polydatin, *trans*-resveratrol, (+)-catechin, (−)-epicatechin, (−)-gallocatechin, quercetin, OH-tyrosol and tyrosol. They corresponded to different non-flavonoids (hydroxybenzoic and hydroxycinnamic acids, stilbenes and phenylethanol analogs) and flavonoids (flavanols and flavonols). The Cabernet Sauvignon GPE showed the highest content of PPs, followed by Bonarda GPE and Aspirant Bouchet (74% and 33% respectively, considering Cabernet Sauvignon as 100%). It is important to mention that there is no information about PPs content for the Bonarda and Aspirant Bouchet GPEs, so the achieved results add knowledge for these grape varieties. Bonarda is the second most cultivated grape variety in Argentina and Aspirant Bouchet is a teinturier grape variety, which has

Table 2
Accuracy, expressed as absolute recoveries (%), and precision, given as standard deviation (SD), of the proposed method for GPE spiked at different concentration levels.

	Recovery (%) ± SD, n = 3 replicates	
	2.5 μg g^{-1}	25 μg g^{-1}
Gallic acid	92.4 ± 5.9	81.2 ± 11.4
(−)-Gallocatechin	85.2 ± 7.0	69.2 ± 9.6
OH-tyrosol	77.9 ± 5.5	80.6 ± 6.6
Caftaric acid	89.8 ± 6.1	80.1 ± 7.8
Tyrosol	82.1 ± 10.7	109.3 ± 10.2
(−)-Epigallocatechin	93.4 ± 7.1	81.3 ± 7.2
(+)-Catechin	110.8 ± 9.6	124.3 ± 10.1
Caffeic acid	102.6 ± 6.3	87.4 ± 6.9
Syringic acid	108.3 ± 9.1	112.9 ± 10.7
(−)-Epicatechin	109.3 ± 7.5	116.6 ± 11.2
p-Coumaric acid	90.6 ± 9.0	74.3 ± 8.9
(−)-Gallocatechin gallate	72.3 ± 8.9	77.8 ± 9.8
Ferulic acid	89.5 ± 11.1	83.2 ± 8.8
Polydatin	112.3 ± 10.1	115.6 ± 5.6
Piceatannol	108.6 ± 9.5	116.9 ± 9.9
Quercetin-3-glucoside	76.8 ± 6.9	70.6 ± 8.1
Kaempferol-3-glucoside	80.2 ± 8.4	72.9 ± 9.5
Trans-resveratrol	114.9 ± 10.6	120.1 ± 8.7
Cinnamic acid	95.9 ± 5.8	103.6 ± 6.3
Quercetin	75.6 ± 12.1	71.8 ± 11.6

presented a 1000% increase of planting in the last 10 years in this country, increasing the interest related to these varieties.

The phenolic acids (gallic, syringic, caffeic, p-coumaric and ferulic) were identified and quantified in all of the analyzed GPEs. By comparison of the average concentrations for the hydroxybenzoic acids, it was observed that syringic acid was the most abundant. For hydroxycinnamic acids, p-coumaric and caffeic acid presented high relative levels in Bonarda. The phenylethanol derivatives OH-tyrosol and tyrosol were quantified in Bonarda and Cabernet Sauvignon GPE, while in Aspirant Bouchet GPE these compounds were not detected.

In the category of non-flavonoids, stilbenes are important compounds because their significant antioxidative properties and nutritional applications. The amounts of stilbenes ranged between 6.9 (*trans*-resveratrol, Aspirant Bouchet GPE) and 27.5 μg g^{-1} (*trans*-resveratrol, Bonarda GPE). In terms of relative abundance of stilbenes in each GPE variety, Aspirant Bouchet presented about 6-times lower content of these compounds. The resveratrol contents reported in the present work were similar to those reported by Casazza et al. for the cultivar (cv.) Pinot Noir (11.6–22.4 μg g^{-1}) (Casazza, Aliakbarian, De Faveri, Fiori, & Perego, 2012) and higher than the reported for Careri et al. for cv. Nero d'Avola (6 μg g^{-1}) (Careri, Corradini, Elviri, Nicoletti, & Zagnoni, 2003).

The flavan-3-ols compounds presented the highest polyphenols concentrations in the studied GPEs. Particularly, the flavanols (+)-catechin and (−)-epicatechin were the most abundant PPs with concentrations ranging between 974.8 and 4397.2 μg g^{-1}. In Bonarda and Cabernet Sauvignon GPE, the (+)-catechin concentrations were higher than those of (-)-epicatechin (between 61% and 69% of total flavonols content), whereas Aspirant Bouchet reported an opposed comportment being (−)-epicatechin the most abundant flavanol. This second behavior was also reported by Escribano-Bailón, Gutiérrez-Fernández, Rivas-Gonzalo, and Santos-Buelga (1992) and Iacopini, Baldi, Storchi, and Sebastiani (2008) for seeds of the Tinta del Pais and Canaiolo red grape varieties, respectively. As well, the distribution between (+)-catechin and (−)-epicatechin (for Bonarda and Cabernet Sauvignon) agrees with that reported by Monrad, Howard, King, Srinivas, and Mauromoustakos (2010) for GPE from Sunbelt grapes (*Vitis labrusca* L.). The prevalence of (+)-catechin has been previously observed in the literature for skins and/or seeds of different red grape varieties (Cheng, Bekhit, McConnell, Mros, & Zhao, 2012; Iacopini et al., 2008; Kammerer, Claus, Carle, & Schieber, 2004; Wang, Tong, Chen, & Gangemi, 2010).

Table 3
Levels of PPs in freeze-dried GPEs. Average concentrations (μg g^{-1} GPE) with their standard deviations, n = 3 replicates.

Analyte	Bonarda	Cabernet Sauvignon	Aspirant Bouchet
Hydroxybenzoic acids			
Gallic acid	250.4 ± 11.2	340.4 ± 25.3	99.6 ± 14.6
Syringic acid	1041.7 ± 77.6	558.9 ± 27.2	843.4 ± 69.8
Total	1292.1	899.3	943.0
Hydroxycinnamic acids			
Caftaric acid	n.d.	n.d.	n.d.
Caffeic acid	83.5 ± 1.0	21.7 ± 2.93	9.9 ± 0.5
p-Coumaric acid	77.0 ± 0.7	21.6 ± 2.3	13.9 ± 1.6
Ferulic acid	24.4 ± 1.4	9.9 ± 0.3	7.9 ± 0.8
Cinnamic acid	n.d.	n.d.	n.d.
Total	184.9	53.2	31.7
Stilbene			
Polydatin	19.7 ± 0.5	11.2 ± 0.5	n.d.
Piceatannol	n.d.	n.d.	n.d.
Trans-resveratrol	27.5 ± 0.6	15.9 ± 0.3	6.9 ± 0.8
Total	47.2	27.1	6.9
Flavanols			
(+)-Catechin	2242.4 ± 124.0	4397.2 ± 507.6	974.8 ± 185.6
(−)-Epicatechin	1553.0 ± 106.4	2069.6 ± 304.3	1302.1 ± 202.8
(−)-Gallocatechin	171.7 ± 3.2	208.1 ± 3.0	89.6 ± 5.4
(−)-Epigallocatechin	n.d.	n.d.	n.d.
(−)-Gallocatechin gallate	n.d.	n.d.	n.d.
Total	3967.1	6674.9	2366.5
Flavonols			
Quercetin-3-glucoside	n.d.	n.d.	n.d.
Kaempferol-3-glucoside	n.d.	n.d.	n.d.
Quercetin	1674.8 ± 200.7	2092.5 ± 113.9	115.4 ± 22.6
Total	1674.8	2092.5	115.4
Other compounds			
OH-tyrosol	13.2 ± 1.4	13.3 ± 0.6	n.d.
Tyrosol	122.7 ± 1.8	45.1 ± 6.9	n.d.
Total	135.9	58.4	–
Total LMW-PPs	7302.0	9804.2	3348.3

n.d., not detected.

With respect to flavonol content of studied GPE, quercetin was the only compound detected and quantified in studied GPEs with concentrations between 115.4 and 2092.5 μg g^{-1}. The quercetin concentrations obtained in this work for red GPEs of Cabernet Sauvignon and Bonarda are higher than the reported by Wang et al. (2010) (200 μg g^{-1}, Muscadine GPE), Hogan, Canning, Sun, Sun, and Zhou (2010) (1600 μg g^{-1}, Norton GPE, higher than Bonarda GPE levels), Careri et al. (2003) (104 μg g^{-1} Nero d'Avola GPE) and Casazza et al. (2012) (287 μg g^{-1} Pinot Noir GPE).

4. Conclusions

A HPLC-MWD method for the simultaneous determination and quantification of twenty polyphenols by using a core–shell column was developed. Under optimized conditions, the resolution of analytes was achieved in about 12 min, with UHPLC performance without build-up pressure. The sensitivity was good enough to assure reliable quantification at levels commonly found in GPEs, with suitable precision and linear response ranges. As well, accuracy of the method was demonstrated when the recovery study was performed over a GPE sample. The applicability of the developed method was verified by the suitable quantification of analytes

in complex GPEs. These facts make the proposed method a convenient alternative because it is sensitive, simple, rapid and compatible with conventional and relatively cheap HPLC systems. Finally, the determination of polyphenol profile of analyzed GPEs contributes to a valuable database for selecting the most appropriate winemaking by-product and grape variety depending on the phenolic compounds required. In this sense, the method could be useful for a high throughput quality control of extracts and to a fast quantification of major PPs in a routine work before the industrial application of obtained GPEs.

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