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CHEMICAL SCIENCES

Antioxidant activity and development of one chromatographic method to determine the phenolic compounds from Agroindustrial Pomace

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Abstract: A chromatographic method consisting of multi wavelength detection for identification of six phenolic acids, one stilbene and five flavonoids in grape and apple pomaces was proposed. Scavenging of DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), reactive oxygen species and reduction of Fe^{3+} to Fe^{2+} using *in vitro* and HPLC-UV-ABTS on-line methods are herein presented. A reversed phase C_{18} coupled with an absorption detector operating at 280, 300, 320 and 360 nm for the benzoic acid derivatives and flavanols; stilbenes; cinnamic acid derivatives and flavonols, were respecctively used. The solvents water, methanol and acetonitrile acidified with acetic acid were evaluated as mobile phase. The optimized chromatographic method presented recoveries ranged from 68 to 130% and from 66 to 130% for grape and apple pomaces respectively. The determination coefficients (R^2) of the 12 compounds were > 0.98. The extracts showed high total phenolic content and exhibits strong capacities to scavenge free radicals and reactive oxygen species. The results obtained by HPLC-ABTS on-line method suggest that pomaces of grape and apple are rich in bioactive compounds and that catechin and epicatechin contribute in a significantly way to the antioxidant activity in both agroindustrial pomaces.

Key words: HPLC-ABTS-on-line, phenolic compounds, antioxidant activity, grape pomace, apple pomace.

INTRODUCTION

Grapes (*Vitis* spp.) and apples (*Malus domestica)* are important fruits in the world because of their organoleptic qualities and also due to production of their derivatives (Alberti et al. 2014, Georgiev et al. 2014). The fruticulture is an important sector of Brazilian agribusiness and Brazil ranks as third-biggest fruit producer in the world with an estimated production in 2016 of 987.059 t and 1.064.708 t for grapes and apples, respectively (Carvalho 2017). Brazilian fruits are very popular abroad due to the quality and sustainability of the sector and the European Union is the main destination of national fruits.

When fruits are processed to produce derivatives, significant amount of by-products are produced and they are still considered waste (Rana et al. 2014) representing problems for disposal in the environment and generating economic losses for agribusiness (Guyot et al. 2003). Apples, grapes and by-products have a complex chemical composition and they are characterized by high contents of phenolic acids, flavonoids and superior phenolics (condensed tannins and proanthocyanidins) (Karling et al. 2017). The phenolic compounds

are secondary metabolites produced by plants as a part of defense mechanisms against UV radiation, pathogen attack and mechanical injury. In humans, these compounds present pharmacological activities as antioxidant (Karling et al. 2017), anti-inflammatory (Brezani et al. 2019) and antimicrobial (Oldoni et al. 2016). The antioxidants can protect the organism from diseases as metabolic disorders and cancer, acting as defense agents by scavenging radicals that are produced in cellular metabolism (Muñoz-González et al. 2014, Alberti et al. 2014).

Reverse-phase chromatography (RP-HPLC) coupled to UV-VIS multi-wavelenght (MWD) detector is an analytical technique widely used for separation, identification and quantification of phenolic compounds in food (Dresch et al. 2014, Fontana & Bottini 2014, Lima et al. 2015) and usually requires optimising a mobile-phase conditions as pH and ionic strenght to achieve a method with lower retention times and suitable retention factors for each analyte (Francisco & Resurreccion 2009). Koleva et al. (2001) optimized a instrumentation using HPLC separation with post column reaction using stable radical solution (ABTS or DPPH) that react with individual compounds and antioxidant activity is measured. Due to complexity of chemical composition of fruits and their by-products, many different antioxidant methods can be used to determine the antioxidant capacity of a matrix. The main methods used are ABTS (2,2-azinobis-(3-ethylbenzothiazoline)-6 sulphonicacid) (Re et al. 1999) and DPPH (2,2-diphenyl-1-picrylhydrazyl) (Brand-Williams et al. 1995) radical scavenging, reducing Fe^{3+} to Fe^{2+} (FRAP) (Benzie & Strain 1996), Oxygen radical absorbance capacity (Ou et al. 2001) and determination of total phenolics with Folin–Ciocalteu reagent (Singleton et al. 1999).

The objectives of this study were to develop a single HPLC method using multiwavelenght detector for simultaneous determination of 12 compounds representatives of phenolic acids, flavonoids and stilbenes in grape and apple pomaces. From developed chromatographic method, the antioxidant activity of each peak was measured using an on-line HPLC–UV-ABTS assay and the total antioxidant capacity of extracts was determined by scavenging of synthetic radicals and reactive oxygen species.

MATERIAL AND METHODS

Chemicals

The reagents potassium persulfate, phosphate buffer, fluorescein disodium, 2,2'-azo-bis (2-amidinopropane) dihydrochloride (AAPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), (+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-Tri(2-pyridyl)-s-triazine(TPTZ), and HPLC analytical standards: caffeic acid, cinnamic acid, ferulic acid, gallic acid, myricetin, p-coumaric acid, quercetin, rutin, (+) – catechin, (-) – epicatechin, vanillic acid and *trans*-resveratrol were obtained from Sigma-Aldrich, St. Louis, MO, USA; acetic acid and the ultrapure solvents for chromatographic assays were purchased from J. T. Baker, Phillipsburg, NJ, USA; Na_2CO_3, Folin-Ciocalteu reagent (Dinâmica Química Contemporânea, Diadema, SP, Brazil) and ultrapure water (Millipore SAS, Molsheim, France).

Sample collection and extraction procedure

The grape (*Vitis labrusca* – Bordeaux variety) and apple (*Malus domestica* – Fuji variety) pomaces samples were obtained in 2015 from wine and juice processing in Paraná and Santa Catarina states, Brazil. The phenolic compounds extraction from pomaces was developed as suggested by Karling et al. (2017) after freeze-drying and

crushing. Initially, 25 mL of ethanol 50% and 65% were added to a flask containing 2 g of grape and apple pomaces respectively. The mixtures were transferred to a water bath at 50°C and 75°C and maintained for 45 and 35 min for grape and apple pomaces respectively. The ethanolic extracts of grape (EEGP) and apple (EEAP) pomaces were cooled, filtered and all extracts and analyses were performed in triplicate.

HPLC analysis and determination of solvent composition

Analysis was developed in a Varian 920 LC HPLC system (Varian Inc., Walnut Creek, CA, USA) equipped with a C_{18} RP column (4.6 x 250 mm, 5 µm particle size) (Microsorb MV-100, Agilent Technologies, Wilmington, DE, USA). For determination of solvent composition were evaluated two compositions of mobile phase. The both compositions used ultrapure water (solvent A) while the organics modifiers evaluated were methanol and acetonitrile (solvent B), all of them acidified with acetic acid (Burin et al. 2014). The first phase involved increasing solvent B (methanol) linearly from 5% to 95% over 65 min (gradient stepness = 1.4%) with a flow rate of 1.0 mL/min, at 30°C. The chromatograms from standard mixtures were complex, thus a series of segmented gradients (GS) and composition of solvent B were evaluated to allow a complete separation of the peaks with good resolution and selectivity. The ideal condition was obtained by using acetic acid: water (2:98 v/v) (solvent A) and acetic acid:acetonitrile:water (2:40:58 v/v) (solvent B) in a gradient mode, starting with 5% solvent B to 20% B in 2 min, 25% B in 15 min, 85% B in 25 min, 85% B in 30 min, followed by flushing and conditioning steps at 36 and 45 min respectively (Table I). The column was maintained at a constant temperature of 30 °C and the flow rate was 1.0 mL min^{-1}.

HPLC method

When all phenolic compounds were eluted with suficient resolution (> 1.5) and selectivity (> 1.0), a grape and apple pomaces extracts was spiked with the mixture of standard compounds to conduct the validation procedure.

Matrix effects (ME) and determination of accuracy

The ME calculated according below equation indicates interferences occuring during LC analysis.

$$ME\ \% = (1 - \text{slope MM curve} \div \text{slope solvent curve}) \times 100$$

where MM: matrix-matched calibration standards.

The accuracy was done by addition/recovery experiments (ICH 2015), where a stock standard solution (100 µg mL^{-1}) containing the 12 compounds was added in grape and apple pomaces extracts at three levels (25%, 50% and 100%). The absolute recoveries (R%) were calculated by the equation: $R\ \% = [(Cs - Cb) \div Ct)] \times 100$, where (Cs) concentration of spiked samples, (Cb) non spiked samples, and (Ct) theoretical concentration added to the sample. The Cs and Cb values were established against calibration curves obtained for matrix-matched standards.

Determination of precision

The precision was assessed by interday studies by analyzing in 5 replicates extracts of grape and apple pomaces spiked with standard mixtures of polyphenols at 30 µg mL^{-1} and results were expressed by coefficient of variation (% CV) of set of data.

Table I. Chromatographic conditions for the determination of phenolic compounds in grape and apple pomace extracts.

Chromatographic conditions	
Injection volume	10 µL
Analytical column	reversed-phase octadecylsilyl (C18) column 4.6 x 250 mm, 5 µm particle size (Microsorb MV 100)
Mobile phase	A (acetic acid:water (2:98 v/v)
	B (acetic acid:acetonitrile:water (2:40:58 v/v)
Mobile phase	First segment: 0 – 2 min: 5 – 20% B
	Second segment: 2 - 15min: 20 – 25 % B
	Third segment: 15 – 25 min: 25 – 85% B
	Fourth segment: 25 – 30 min: maintained at 85% B
	Flushing step: 30 – 36 min: 85 – 95% B
	Conditioning step: 36 – 45 min: 95 -5% B
Flow rate	1.0 mL/min
Column Temperature	30°C
Scanning range	200-400 nm
Detection wavelenght	Benzoic acid derivatives and flavanols: 280 nm
	Cinnamic acid derivatives: 320 nm
	Flavonols: 360 nm
	Stilbenes: 300 nm

Determination of Limit of Detection (LOD) and Limit of quantification (LOQ)

The detection and quantification limits were calculated based on (3 x SD)/s and (10 x SD) /s, respectively, where s is the slope of the calibration curves prepared in matrix-matched and SD is the standard deviation of the intercept in the standard curve (ICH 2015). For each standard was used a specific analytical range described in Table II.

Chromatographic analysis of phenolic compounds in grape and apple pomaces extracts

The identification of individual phenolics of extracts have been done by comparing retention times and absorption in UV spectra. For quantification purposes the peak areas obtained using matrix-matching standards were used and a broad analytical ranges for standard calibration curves were adopted to ensure a wide range of quantification. All tests were conducted in triplicate and 10 µL were injected in equipment.

Table II. Validation parameters for the determination of phenolic compounds in grape and apple pomaces extracts.

Analyte	tr (min)	λ (nm)	Linear range (μg mL^{-1})				Precision (RSD %) Interday			Recovery (%)		LOD/ LOQ (μg mL^{-1})	
			Grape matrix	R^2	Apple matrix	R^2	Grape matrix	Apple matrix	Solvent-based standards	Grape matrix	Apple matrix	Grape matrix	Apple matrix
Gallic acid	5.65	280	1.8-30	0.9993	0.5-8.0	0.9997	0.7	2.0	0.4	105	109	0.6/2.1	0.1/0.2
(+)-Catechin	11.8	280	5.0-80	0.9959	0.5-8.0	0.9962	1.9	9.7	0.3	120	130	3.2/7.0	0.4/1.3
Vanillic acid	14.4	280	1.8-30	0.9979	5.0-8.0	0.9999	1.6	1.2	0.7	121	130	1.0/3.2	0.05/0.2
Caffeic acid	15.2	320	1.8-30	0.9995	0.5-8.0	0.9994	1.9	0.7	5.1	114	66	0.6/2.1	0.05/0.2
(-)-Epicatechin	18.3	280	5.0-80	0.9779	5.0-80	0.9981	2.3	2.4	2.2	130	90	0.1/0.2	2.6/8.8
p-Coumaric acid	22.6	300	1.8-30	0.9995	0.5-80	0.9999	0.6	0.3	0.7	73	113	0.2/0.6	0.02/0.08
Ferulic acid	24.4	320	5.0-80	0.9992	0.5-8.0	0.9998	2.5	1.3	1.3	68	86	0.8/2.8	0.3/1.2
Rutin	25.0	360	1.8-30	0.9963	5.0/80	0.9995	1.4	1.1	0.4	109	115	0.4/1.3	0.4/0.4
Salicylic acid	26.5	300	5.0-80	0.9996	5.0-80	0.9965	2.9	12	4.0	75	105	1.0/3.3	0.4/1.4
Myricetin	27.8	360	1.8-30	0.9998	0.5-8.0	0.9964	1.6	11	8.0	68	104	0.3/0.9	0.1/0.5
trans-resveratrol	28.5	300	0.5-8.0	0.9992	0.5-8.0	0.9995	1.2	0.4	0.6	68	119	0.1/0.4	0.1/0.3
Quercetin	30.5	360	3.7-30	0.9920	0.5-8.0	0.9981	3.5	2.4	5.0	69	118	2.4/8.0	0.4/1.4

On-line HPLC–UV–ABTS radical scavenging activity analysis

On-line HPLC–ABTS assay was conducted as reported by Koleva et al. (2001). HPLC separation was performed as described in Table I. The analytes separated and detected by an MWD detector, reacted in a post-column reaction coil (15 m × 0.25 mm i.d. PEEK tubing) at a flow rate of 0.8 mL min^{-1} with radical ABTS and the bleaching was detected as a negative peak at 734 nm. Results were expressed in TEAC (equivalent to Trolox) (R^2 = 0.9968).

Total Phenolic Content (TPC)

The phenolic content was determined as described by Singleton et al. (1999). The mixture was composed by 0.5 mL of EEAP 3.2 g L^{-1} or EEGP 1.6 g L^{-1}, 2.5 mL of Folin-Ciocalteau diluted (10 g L^{-1}) and, after five minutes, it was added 2.0 mL of sodium carbonate (Na_2CO_3) 40 g L^{-1}. The tube with reaction mixture was incubated in the dark for 2 hours at room temperature and then, the absorbance (740 nm) was measured using a spectrophotometer (model UV-VIS lambda 25, Perkin Elmer). Gallic acid was used as reference at concentrations from 10 to 80 mg L^{-1} and the results were expressed as mg GAE $g^{-1}$of dry sample (GAE: gallic acid equivalents).

Scavenging of DPPH, ABTS and Reactive Oxygen Species (ROS)

Antioxidant capacity using 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging method

DPPH free radical scavenging capacity was determined according to Brand-Williams et al. (1995) method. A volume of 0.5 mL of EEGP (0.4 g L^{-1}) or EEAP (0.4 g L^{-1}) were added to 0.3 mL of DPPH radical solution 0.5 mmol L^{-1} in ethanol and 3 mL of ethanol. The mixture was incubated for 45 min in the dark at room temperature and the absorbance was measured at 517 nm using a spectrophotometer. The standard trolox (15 to 100 µmol L^{-1}) was used for calibration curve and the results were expressed as µmol of Trolox g^{-1} of dry sample (µmol Trolox g^{-1}).

Antioxidant activity using 2,2-azinobis-(3-ethylbenzothiazoline)-6 sulphonicacid) (ABTS) method

The antioxidant activity using the ABTS radical was done as suggesting by Re et al. (1999). Initially 7 mmol L^{-1} of ABTS solution reacted with 140 mmol L^{-1} of potassium persulfate and incubated in the dark at 25 °C for 16 hours. The radical ABTS formed was diluted using ethanol until absorbance reached at 0.700 ± 0.200 at 734 nm. The reaction mixture was produced by 3.0 mL of the ABTS solution, and 30 µL of EEGP (0.4 g L^{-1}) or EEAP (0.4 g L^{-1}) and after 6 min the absorbance was measured in a spectrophotometer at 734 nm. The calibration curve was plotted using concentrations that varied from 100 to 2000 µmol L^{-1} of Trolox. The results were expressed in µmol of TEAC g^{-1} of dry sample (TEAC: Trolox equivalent antioxidant capacity).

Ferric Reducing Antioxidant Potential (FRAP) method

The antioxidant activity by the FRAP method was proposed by Benzie & Strain (1996). The FRAP reagent was obtained from a mixture of 25 mL of acetate buffer (0.3 mol L^{-1}), 2.5 mL of a solution of TPTZ (10 mmol L^{-1}) and 2.5 mL of $FeCl_3$ (20 mmol L^{-1}). An aliquot of 90 µL of the EEGP (1.6 g L^{-1}) or EEAP (1.6 g L^{-1}) was added to 3 mL of FRAP reagent. The solution was homogenized and mantained for 30 min in water bath at 37 °C and then the absorbance was measured in a spectrophotometer at 595 nm. The blank was a solution of FRAP reagent and the calibration curve was made by using aqueous solutions

of ferrous sulphate (100 - 2000 µmol L^{-1}). The results were expressed as µmol of Fe^{2+} g^{-1} of dry sample.

Oxygen Radical Absorbance Capacity (ORAC) assay

Peroxyl radical ($ROO^{\cdot}$) scavenging ability was evaluated using methodology described by Ou et al. (2001). The reaction mixture was composed by 30 µL of the control, standard or EEGP (111 µg mL^{-1}) and EEAP (136 µg mL^{-1}), 60 µL of 508.25 nmol L^{-1} fluorescein disodium, and 110 µL of a solution of 76 mmol L^{-1} AAPH in a microplate. A solution of potassium phosphate buffer 75 mmol L^{-1} (pH 7.4) was used as a blank and to dilute the extracts. The reaction occured at 37°C and the emissions were measured every minute for 2 h at 485 nm (excitation) and 528 nm (emission) using a microplate reader SpectraMax® M3. Trolox was used at concentrations ranging from 12.5 to 400 µmol L^{-1} as standard and the results were expressed as mmol Trolox equivalents per g of sample (mmol TEAC g^{-1}).

RESULTS AND DISCUSSION

Exploratory gradient elution

Methanol and acetonitrile were evaluated as mobile phase using gradient steepness and both were acidified with acetic acid for control of retention time and shape of signal. The assays were started with the protic solvent methanol because it is weaker (ε° = 1.0) and have shown lower cost than acetonitrile (ε° = 3.1). However, the signals obtained did not show suitable values for selectivity and resolution (data not showed) with coelution of six compounds and a total running time of 65 minutes. In addition to the composition and gradient of the mobile phase, changes in the flow rate and temperature of the column were also evaluated.

To alter the selectivity, the acetonitrile solvent was tested. The acetonitrile has been used by various authors to separate phenolic compounds (Burin et al. 2014, Fontana & Bottini 2014) and is currently the most used solvent in high-performance liquid chromatography, since it presents advantages like low viscosity and low cut off in the ultraviolet region. A mobile phase with increasing the concentration of water in the elution mixture was evaluated. This alteration decreases the elution strength of the mobile phase, which allows more interaction of compounds with the nonpolar stationary phase C_{18}.

To reduce exposition of stationary phase to high content of water, the initial solvent B composition started at 5% (Neue & El Fallah 1997). An initial GS (gradient stepness) of 7.5% was used to allow a rapid gradient before elution of compounds with composition of solvent B increasing from 5% to 20% in 2 min (first segment). A slow GS of 0.38%, with solvent B increasing from 20% to 25% in 13 min was applied as second segment and finally a GS of 6% was used as third segment to elute that components with less polar characteristics. All compounds were eluted in 35 min and enables detection of 12 phenolic compounds with good selectivity (α > 1) and resolution (Rs > 1.5) (Figure 1).

HPLC method

Determination of Matrix effect (ME) and linear range

Seven-point standard curves in solvent and matrix for each phenolic compound were plotted by relating peak area vs. concentration of polyphenols for each compound. The calibration curves were linear from 0.5 to 80 mg L^{-1} and were obtained with R^2 higher than 0.992

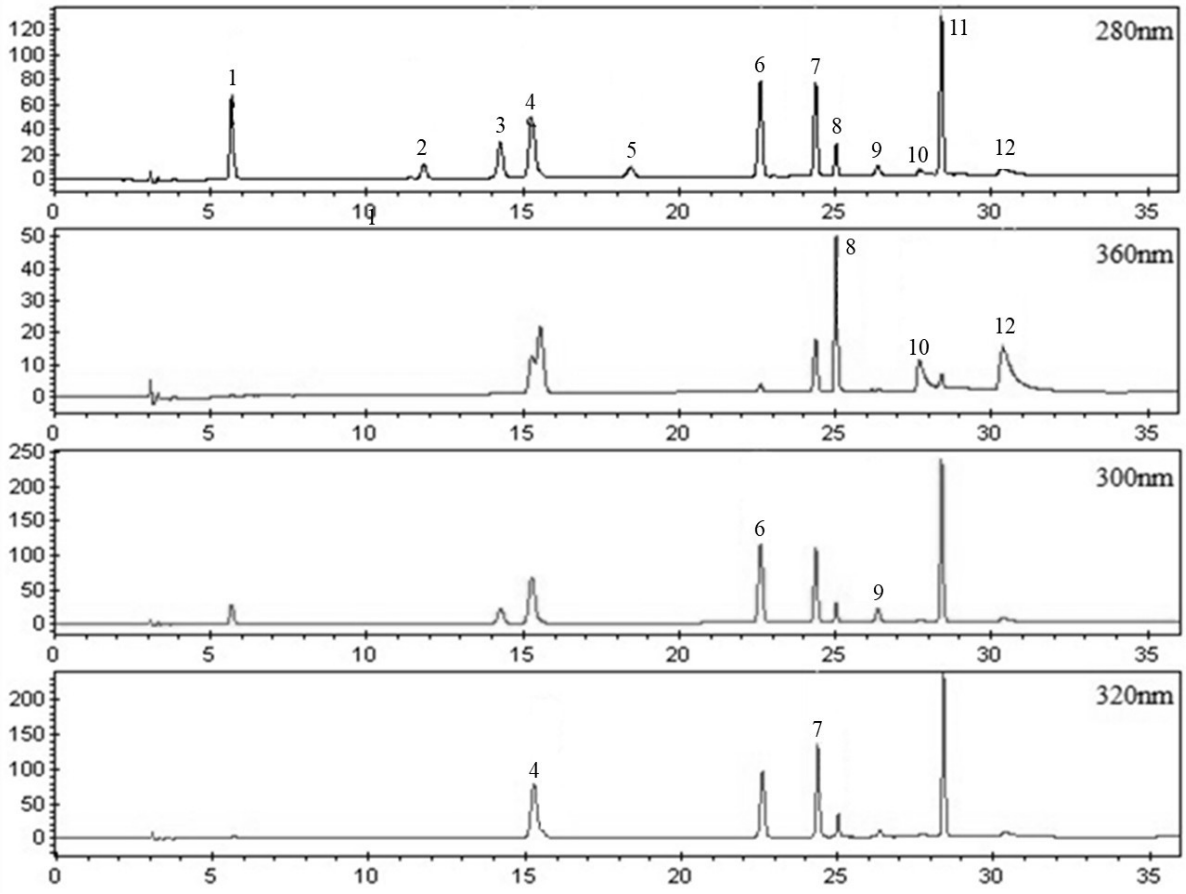

Figure 1. Chromatograms of mixture solvent standard at 30 µg mL^{-1} in 280, 300, 320 and 360 nm. 1. gallic acid; 2. catechin; 3. vanillic acid; 4. caffeic acid; 5. epicatechin; 6. coumaric acid; 7. ferulic acid; 8. rutin; 9. salicylic acid; 10. myricetin; 11. resveratrol; 12. quercetin.

with the excpetion of epicatechin in grape matrix (see Table II). All R^2 values calculated were in accordance with the International Conference of Harmonization (ICH 2015).

Minor matrix effects are observed if calculated values ranged from –20 to +20% (Fontana & Bottini 2014). For both pomaces extracts evaluated, about 30% of the investigated analytes did not shown a significant ME. Thus, matrix-matched standards were used as technique to quantification of compounds.

Determination of accuracy

Recoveries of grape and apple pomaces ranged from 66% to 130% (Table II) for all phenolic compounds evaluated. Recommended average recovery of an analyte ranges from 70% to 125% (ICH 2015). For apple pomace the lowest recovery was obtained for caffeic acid (66%) and the highest recoveries (130%) were verified for vanillic acid and catechin. Recoveries for gallic acid, quercetin, *trans*-resveratrol and *p*-coumaric acid were 109%, 118%, 119% and 113% respectively. For grape pomace, the ferulic acid, miricetyn and *trans*-resveratrol showed lowest recoveries, next to 67% while highest recovery of 130% was obtained for epicatechin. Recoveries for gallic acid and catechin were 105% and 120% respectively.

Determination of precision

For grape and apple pomaces spiked with standards (30 µg mL^{-1}) was obtained a coefficient of variation (CV) ranged from 0.6 to 3.5% and 0.3 to 12% respectively (Table II), while the calculated CV for standards in solvent ranged between 0.3% to 8.0%. These values are considered adequate to determine quantitatively polyphenols in pomaces since aceptable limits up to 20% of variation are suggested by ICH (2015) for determination of low-level constituents. The results of CV were similar to that reported by Fontana & Bottini (2014) in a study to determine polyphenols in red and white wine and the authors obtained CV ranged from 2.3% (gallic acid) to 11.6% (ferulic acid).

Determination of limit of detection and limit of quantification

For solvent-based standards LOD ranged from 0.05 to 1.45 µg mL^{-1} while LOQ varied from 0.15 to 4.88 µg mL^{-1} (data not showed). For grape-matched standards the lowest LOD and LOQ were obtained for epicatechin (0.1 and 0.2 µg mL^{-1} respectively) while lowest LOD and LOQ for apple-matched standards were 0.02 and 0.08 µg mL^{-1} respectively for coumaric acid. The compounds catechin and epicatechin showed higher limits for grape and apple matched standards respectively (Table II).

Phenolic profile of grape and apple pomaces by HPLC-MWD

In grape pomace extract (Figure 2 a, b , Table III) were identified in minor concentration gallic acid (9.10 mg 100 g^{-1}) and quercetin (32.90 mg 100 g^{-1}) and in major concentration flavonol catechin (82.8 mg 100 g^{-1}), followed by myricetin and epicatechin. Farhadi et al. (2016) studied grapes and identified compounds with antioxidant activities mainly flavonoids as catechin, epicatechin, rutin and quercetin flavonoids.

For the apple pomace extract (Figures 3a, b), the flavonoids catechin (18.5 mg $100g^{-1}$) and epicatechin (58.9 mg $100g^{-1}$) were identified in high concentration while rutin (5.71mg $100g^{-1}$) was found in lower concentration (Table III).

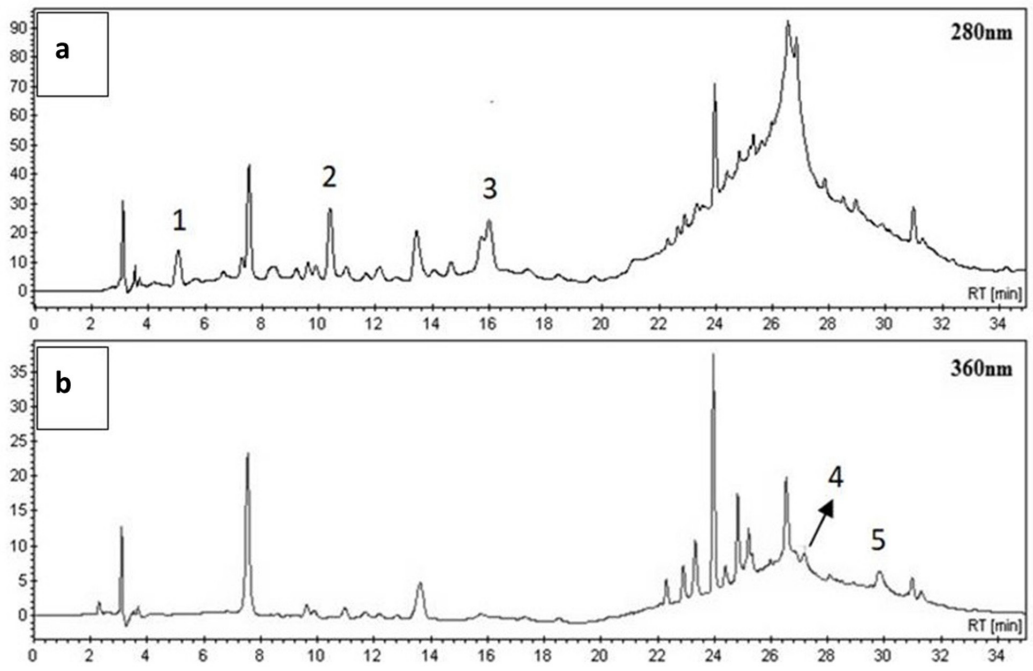

Figure 2. HPLC profile of grape pomace extract at 280 nm (a) and 360 nm (b) (mAU x time). Peaks: 1. gallic acid; 2. catechin; 3. epicatechin; 4. myricetin; 5. quercetin;

Table III. Phenolic profile of grape and apple pomace extracts using optimized HPLC-DAD methodology.

Extract	Compounds	Regression equation	Concentration (mg $100g^{-1}$)
EEGP	Gallic Acid	y=0,2790x+0,4560	9.10
	(+)-Catechin	y=0,0669x+0,0736	82.8
	(-)-Epicatechin	y=0,0682x+0,0561	41.7
	Myricetin	y=0,0713x-0,1344	71.7
	Quercetin	y=0,2017x+0,1458	32.9
EEAP	(+)-Catechin	y=0,0669x+0,0736	18.1
	(-)-Epicatechin	y=0,0682x+0,0561	58.9
	Rutin	y=0,1547x+0,2600	5.71

Regression equations obtained from analitical standards in matrix.

Alberti et al. (2014) found similar results when they investigated the Gala apple variety and identified catechin and epicatechin as the most common phenolic compounds.

Total phenolic compounds and antioxidant activity of apple and grape pomaces

For a broader understanding of the antioxidant properties of the material being studied, the antioxidant potential of the grape and apple pomaces were assessed by using *in vitro* methods: the Total Phenolic Content (TPC), scavenging of DPPH, ABTS and reactive oxygen species and FRAP methods (Table IV).

Through the Tukey test differences were observed among the assessed pomaces extracts in all the antioxidant activities evaluated. It can be concluded that grape pomace presented twice the activity of apple pomace for the ABTS and DPPH radical scavenging assays and four times the content of total phenolic compounds.

The antioxidants activities observed in grape and apple pomaces may be related to the presence of the monomers (+)-catechin and its isomer (-)-epicatechin, determined in major concentrations in both extracts. These molecules as well as oligomeric and polymeric forms (Manach et al. 2005) and have many biological properties (Georgiev et al. 2014, Pizzolitto et al. 2013).

In their studies Rockenbach et al. (2011) assessed the antioxidant activities and content of phenolic compounds of different varieties of red grape pomaces. The Cabernet sauvignon pomace presented the highest content of total phenolic compounds and the highest antioxidant. For the Isabel variety, the results were 32.62 mg GAE g^{-1} for total phenolic compounds and 188.02 µmol TEAC g^{-1}, for the DPPH radical scavenging method, similar to the findings of the present study.

Studies developed by Tornour et al. (2015) with grape pomaces from Portugal showed ORAC values of 1579 µmol TEAC g^{-1} that are similar to those obtained in this study. When compared to the USDA ORAC database (USDA 2010), total ORAC of grape and apple pomaces were greater than grape, apples and their juices listed in database. As samples evaluated in this study are primarily by-products of grape and apple processing, this results suggests that these low value materials could be used as substitutes for synthetic antioxidants.

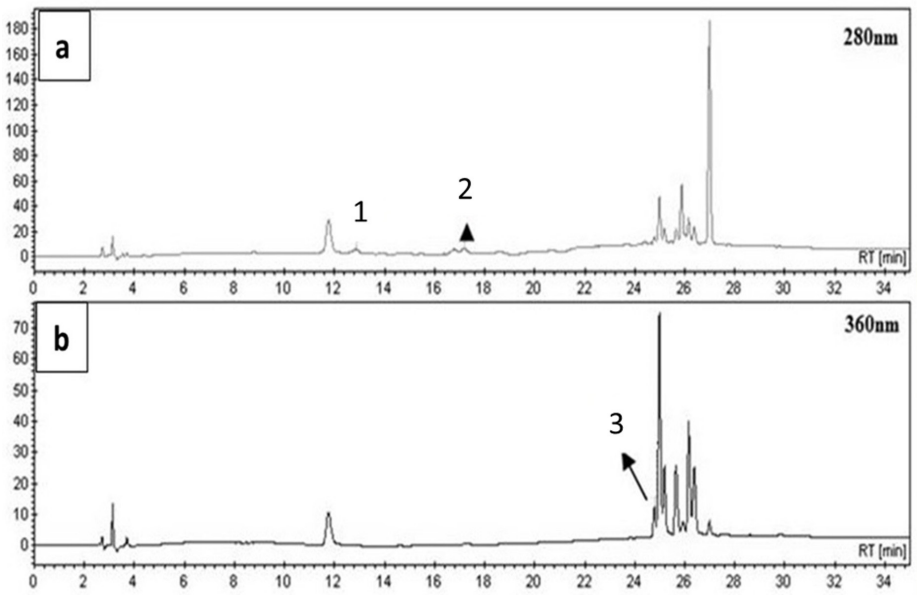

Figure 3. HPLC profile of apple pomace extract at 280 nm (a) and 360 nm (b) (mAU x time). Peaks: 1. catechin; 2. epicatechin; 3 . Rutin.

Differences in the TPC and antioxidant activities can occur due to variations in the analyzed species and cultivars and in weather conditions. Differences in the extraction methods can also influence the content of these compounds (Karling et al. 2017). The results obtained showed that the residue *Vitis labrusca* grape contains equal or higher content of antioxidant activity and total phenolic compounds than *Vitis vinífera*, suggesting to be a good source of natural compounds for pharmaceutical, food and cosmetic industries.

Despite the apple pomace has presented lower values for total phenolic compounds and antioxidant activities, it can also be considered a by-product with antioxidant potential, which can be utilized in the recovery of natural antioxidant resources, under the form of polyphenols. Studies carried out by Wijngaard & Brunton (2010) assessed antioxidant activity, by means of the DPPH method, and the content of phenolic compounds in apple pomace. Maximum extraction of phenolic compounds (1092 mg GAE $100g^{-1}$) and antioxidant activity by DPPH (449 mg Trolox $100g^{-1}$) method was obtained with 56% ethanol at 80°C during 27 min.

Antioxidant activity by on-line HPLC-UV-ABTS

The chromatogram obtained for grape pomace (Figure 4) showed four signs that presented antioxidant activity, especially signals related to catechin and epicatechin, suggesting a high antioxidant potential in these products that is closely linked to these flavonoids. All of the signs that presented antioxidant capacity were detected at 734 nm, a wavelength that is specific for the identification of ABTS radical scavenging.

For apple pomace (Figure 5), the identified compounds catechin, epicatechin and rutin presented the highest response to the on-line antioxidant activity. In addition, a signal with retention time of approximately 19 minutes was observed. It was not identified, due to the lack of patterns; however, it also presented a high level of activity. It can be pointed out that the major signal that eluted with a retention time close to 30 minutes did not demonstrate significant activity through the on-line ABTS method.

The on-line HPLC–UV–ABTS method showed selectivity and simplicity for determination of antioxidant activity when used for identification of antioxidant individual components of grape and apple pomaces.

Table IV. Antioxidant activity and Total Phenolic Content of grape and apple pomaces extracts.

	TPC mg GAE g^{-1}	DPPH µmol TEAC g^{-1}	ABTS µmol TEAC g^{-1}	FRAP µmol $FeSO_4$ g^{-1}	ORAC mmol TEAC g^{-1}
EEGP	33.9±1.2 a	212.7±15.6 a	445.1±31.1 a	589.1±8.0 a	1.64±0.02* a
EEAP	8.32±0.06 b	123.8±9.24 b	212.2±12.7 b	174.2±4.6 b	0.33±0.02* b

The same letter in the column, are not significantly different at the level of 0.05 according to ANOVA. The values are average ± standard deviation (*n* = 4). TPC = Total Phenolic Content; TEAC = Trolox Equivalent Antioxidant Capacity; * freeze-dried extract diluted in a phosphate buffer.

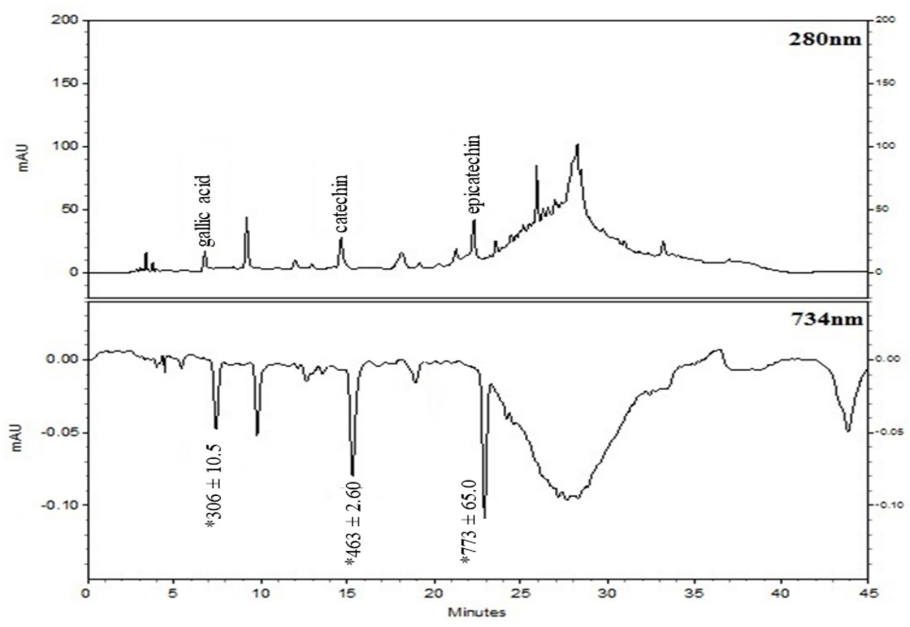

Figure 4. HPLC profile of grape pomace extract at 280 nm (a) and HPLC-UV-ABTS *on-line* at 734 nm (b). *µmol TEAC g^{-1}.

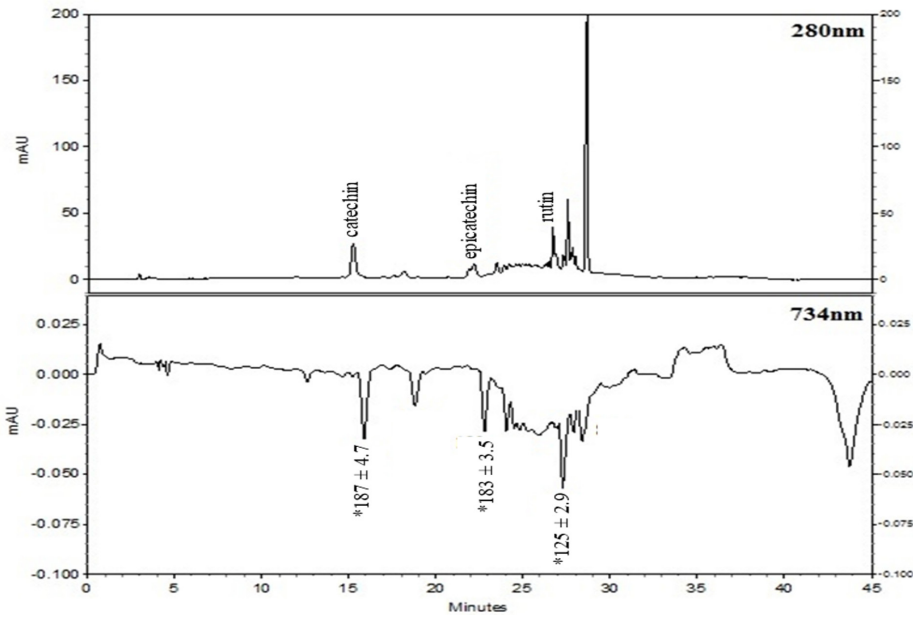

Figure 5. HPLC profile of apple pomace extract at 280 nm (a) and HPLC-FR-$ABTS^{\cdot+}$ *on-line* at 734 nm (b). *µmol TEAC g^{-1}.

CONCLUSION

A chromatographic method for separation, identification and quantification of 12 polyphenols from grape and apple pomaces extracts with good resolution, precision and accuracy was carried out. The composition (2% acetic acid in water and acetonitrile) and gradient of mobile phase optmized can be used in a HPLC system coupled to a octadecylsilane stationary phase and multiwavelenght detection. The antioxidant capacity of pomaces extracts was measured by on-line HPLC assay and *in vitro* methods. The grape pomace extract showed higher antioxidant activity in all assays evaluated and gallic acid, catechin and epicatechin were identified as compounds that contribute with potential activity.

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T.L.C Oldoni designed the study and interpreted the results. R. C. Silva developed assays and interpreted results. S. T. Carpes interpreted results of antioxidant activity. A.P.Massarioli developed antioxidant activity by ORAC method. S.M. Alencar interpreted the results of antioxidant by HPLC on-line method.