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Antioxidants and bioactivities of free, esterified and insoluble-bound phenolics from berry seed meals

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ABSTRACT

Phenolic compounds present in the free, soluble ester and insoluble-bound forms of blackberry, black raspberry and blueberry were identified and quantified using high-performance liquid chromatography-diode array detection-electrospray ionisation multistage mass spectrometry. The total phenolics, scavenging activity against hydroxyl and peroxyl radicals, the reducing power and chelating capacity were, in general, in the decreasing order of blackberry > black raspberry > blueberry. Amongst fractions, the order was insoluble-bound > esterified > free. These trends were the same as those found against copper-induced LDL-cholesterol oxidation and supercoiled plasmid DNA strand breakage inhibition induced by both peroxyl and hydroxyl radicals. Extracts were found to contain various levels of phenolic compounds that were specific to each berry seed meal type. Berry seed meals should be considered as a good source of phenolics with potential health benefits. Their full exploitation may be helpful for the food industry and consumers.

1. Introduction

The consumption of fresh fruits and vegetables has continued to increase, primarily due to a better understanding about the association of their intake with a lower incidence of cardiovascular disease, cancer, degenerative diseases, and other chronic ailments. Oxidative stress, which is induced by free radical attack on cellular components by reactive oxygen species (ROS), has a major role in the development of many degenerative diseases. As a result, antioxidants protect against oxidative stress and, therefore, they are considered important in reducing the initiation and progression of these diseases. Endogenous antioxidant systems play a crucial function in combating oxidative stress, but dietary antioxidants are also important (Cotran, Kumar, & Collins, 1999; Shahidi & Ambigaipalan, 2015).

Fruits and vegetables are valuable sources of phytochemicals, such as carotenoids, vitamins C and E, folate, phenolic and thiol compounds. The relationship between increased fruit and vegetable intake and lower risk of chronic diseases could be attributed to their antioxidant activity. Unlike animal dietary sources, plant-based diets contain many simple phenolic and polyphenolic compounds that possess significant antioxidant properties. In plants, the polyphenolic compounds provide several eco-physiological functions, involving chemical and physiological defense responses, and they are varied in their structures and chemical properties. As components in the human diet, phenolic compounds are considered to act as antioxidants directly, or to affect the production or function of other antioxidant compounds in the body (Halvorsen et al., 2002; Prior, 2014).

Antioxidant efficiency may be evaluated by several methods, which stems from their capacity in scavenging radicals by single electron transfer (SET) and hydrogen atom transfer (HAT) mechanisms (Leopoldini, Marino, Russo, & Toscano, 2004). These assays vary in terms of their principles and experimental conditions. Therefore, each assay provides an estimate of antioxidant efficiency that is based on the type of method selected and experimental conditions employed. Thus, the use of different methods assists in identifying variations in the compounds extracted from plant sources.

In the production of juices, jellies and jams from berries, most processors remove the berry seeds prior to processing. The seeds are commonly used as a source of specialty oil and the leftover meal (flour) is rich in bioactive ingredients (Helbig, Böhm, Wagner, Schubert, & Jahreis, 2008). Unlike oil seeds, berry seed meals have not commonly gained much attention as antioxidant sources, this could be attributed to their lack of popularity and varied commercial applications. Thus, it would be beneficial, if they could be employed as a source of natural food additives and ingredients in enhancing the complete utilisation of the seeds.

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Therefore, the aims of this study were to determine the total phenolics of selected berry seed meals, identify the various phenolic compounds in each sample by high performance liquid chromatography-diode array detection-electrospray ionisation-tandem mass spectrometry (HPLC-DAD-ESI-MS^n) and evaluate the potential health benefits of these byproducts to deactivate ROS and metal ions, followed by testing of their efficacy in biological model systems.

2. Materials and methods

2.1. General

The seed meal of blackberry, black raspberry, and blueberry samples were provided by the Fruit Smart Company, Grandview, WA, USA. Samples were packed immediately in vacuum bags and stored in a freezer at −20 °C.

Trolox(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Acros Organics (Fair Lawn, NJ, USA). Organic solvents and reagents, such as methanol, acetone, acetonitrile, formic acid, hydrochloric acid, sodium hydroxide, and sodium carbonate, were purchased from Fisher Scientific Co. (Nepean, ON, Canada). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), Folin and Ciocalteau's phenol reagent, and all phenolic compound standards with a purity of ⩾96%, sodium hydroxide, hydrogen peroxide, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), ferrous sulphate, fluorescein, potassium ferricyanide, trichloroacetic acid, ferrous chloride, ferrozine, ferric chloride, human LDL-cholesterol, $CuSO_4$, sodium chloride, mono- and dibasic sodium and potassium phosphates, ethylenediaminetetraacetic acid (EDTA), deoxyribonucleic acid (DNA) of pBR 322 (*Escherichia coli* strain RRI), agarose, Tris acetate, bromophenol blue, xylene cyanol, and glycerol were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada).

2.2. Preparation of samples

The oil from the berry seed meals were commercially removed but there are still some residual oil remaining in the berry seed meals. Therefore, an extra effort is needed to remove them from the samples. Thus, all samples were defatted by blending the ground material with hexane (1:5, w/v, 5 min) in a Waring blender (Model 33BL73, Waring Products Division, Dynamic Corp of America, New Hartford, CT, USA) at room temperature (24 °C), three times. The resultant extracts were combined and filtered through Whatman #1 filter paper with suction using a Buchner funnel. Defatted samples were air dried for 12 h and vacuum packaged in polyethylene pouches and kept at −20 °C until used.

2.3. Extraction of phenolics

Extraction of phenolic acids and minor amounts of flavonoids, proanthocyanidins and anthocyanins from the defatted berry seed meals were performed according to the method outlined by Krygier, Sosulski, and Hogge (1982), and as explained by Naczk and Shahidi (1989). Soluble phenolics were extracted using methanol–acetone–water (7:7:6, v/v/v), acetone–water (80: 20, v/v), methanol–water (70:30, v/v), or water, and each was used separately. About 5 g of each sample were extracted with 75 ml of each solvents. These samples were ultrasonicated for 20 min at 30 °C, then centrifuged for 5 min at 4000*g*. After centrifugation, the upper layers were combined and the extraction operation was repeated twice. The collected supernatants were examined for free phenolics and soluble phenolic esters, and the solid residue was stored for evaluation of insoluble-bound phenolics. The organic solvent from combined extracts was removed under vacuum at 40 °C using a rotary evaporator (Rotavapor model 461, Büchi, Flawil, Switzerland). Before extraction with hexane, the aqueous phase was acidified to pH 2 using 3 M HCl and centrifuged to separate any precipitates. Extraction of free phenolics was carried out 3 times with an equal volume of diethyl ether–ethyl acetate (1:1, v/v). Diethyl ether–ethyl acetate layer was filtered through anhydrous sodium sulphate, using a No. 1 Whatman filter paper, and combined, evaporated to dryness, and dissolved into 5 ml of HPLC grade methanol. The esters present in the aqueous phase were hydrolysed with 4 M NaOH for 4 h. The pH of the hydrolysate was adjusted to 2 and the liberated phenolics were extracted with diethyl ether–ethyl acetate (1:1, v/v), evaporated to dryness and subsequently dissolved in 5 ml methanol. The solid residue obtained following the extraction of the soluble phenolics was dissolved in 40 ml of 4 M NaOH whilst stirring for 4 h. The mixture was then adjusted to pH 2, centrifuged and the insoluble-bound phenolics were extracted with diethyl ether–ethyl acetate (1:1, v/v) in the same manner as explained above.

2.4. Determination of total phenolic content

The total phenolics were estimated according to the method of Singleton and Rossi (1965). The Folin and Ciocalteau's phenol reagent (0.5 ml) was mixed with 0.5 ml of methanolic extracts in a centrifuge tube, and 1 ml of saturated sodium carbonate (75 g/L) was added to each tube, followed by adjusting the volume to 10 ml with distilled water. The contents were allowed to stand for 45 min at ambient temperature (23 °C). The absorbance was read at 725 nm. The total amount of phenolic compounds was expressed as mg of gallic acid equivalents (GAE) per gram of defatted sample.

2.5. HPLC-DAD-ESI-MS^n analysis

In order to identify the predominant phenolic compounds in the berry seed meal samples, high performance liquid chromatography-diode array detection-electrospray ionisation multistage mass spectrometry (HPLC-DAD-ESI-MS^n) was used (Agilent Technologies, Palo Alto, CA, USA). A slightly modified version of the method described by Määttä, Kamal-Eldin, and Törrönen (2003) and Wu and Prior (2005) was used. Analytical separation of the phenolic compounds was carried out on a Supelcosil LC-18 column (250 × 4.6 mm inner diameter, 5-μm particles, Supelco, Bellefonte, PA, USA). The column oven temperature was maintained at 25 °C. Mobile phases consisted of formic acid (A) and acidified water containing 0.1 M acetonitrile (B). The elution conditions were as follows: 0 min, 100% B; 5 min, 90% B; 35 min, 85% B; 45 min, 60% B; 50 min, 60% B; 55 min, 100% B; 65 min, 100% B and then held for 1 min before returning to the initial conditions. The flow rate was 0.5 ml/min and the wavelengths of detection were 280 and 520 nm. Peak identification of unknown compounds of interest in this study was performed by matching the retention times of unknown compounds with external standards, when available. HPLC-ESI-MS^n analysis was carried out as described by de Camargo, Regitano-d'Arce, Biasoto, and Shahidi (2014a) under the same conditions as described above using an Agilent 1100 series capillary liquid chromatography/mass selective detector (LC/MSD) ion trap system in electrospray ionisation (ESI) in the negative and positive mode. The data were acquired and analysed with an Agilent LC/MSD software. The scan range was set from 50 to 1200 m/z, using smart parameter setting, drying nitrogen gas at 350 °C, flow 12 L/min, and nebulizer gas pressure of 70 psi.

2.6. Hydroxyl radical scavenging activity

The capacity of phenolic compounds in scavenging hydroxyl radicals formed by Fenton reaction was examined by electron paramagnetic resonance (EPR) spectroscopy, using the method

previously reported (de Camargo, Vidal, Canniatti-Brazaca, & Shahidi, 2014b). The hydroxyl radical was generated through Fe (II)-catalysed Fenton reaction and spin trapped with DMPO. The resultant DMPO–OH adduct was detected using an EPR spectrometer. Extracts (200 μl) were mixed with 200 μl of 10 mM H_2O_2, 300 μl of 17.6 mM DMPO, and 200 μl of 0.1 mM $FeSO_4$. All solutions were prepared in deionised water. After 1 min, the EPR spectrum of the mixture was recorded at 5.02×10^2 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.000 G sweep width, 3495.258 G centre field, 5.12 s time constant, 9.795 GHz microwave frequency, 86.00 kHz modulation frequency, and 1.86 G modulation amplitude. Different concentrations (200–2000 mM) of Trolox were used to prepare the standard curve. Hydroxyl radical scavenging capacities of the extracts were calculated using the following equation:

$$\text{Hydroxyl radical scavenging capacity } (\%) = 100 - (\text{EPR signal intensity of samples with extracts} / \text{EPR signal intensity for control}) \times 100.$$

The results were expressed as μmol of Trolox equivalents/g of defatted sample.

2.7. Measurement of oxygen radical absorbance capacity (ORAC)

The ORAC assay was carried out using a Fluostar Optima microplate reader (BMG Labtech, Durham, NC, USA). The internal wells of a 96-well Costar 2650 black plate (Nepean, ON, Canada) were used to perform the ORAC assay. Twenty microlitres of appropriately diluted extract, blank, or Trolox were mixed with 200 μl of 0.11 μM fluorescein solution directly in the microplate and the plate was incubated in FLUOstar at 37 °C for 15 min. Subsequently, 75 μμl of AAPH was injected into each well to initiate the reaction. The plate was shaken for 4 s, and the fluorescence was determined and recorded every min for 60 min with an excitation and emission wavelengths of 485 and 520 nm, respectively. Different concentrations (6.25–100 mM) of Trolox were used to prepare the standard curve. The ORAC values were reported as μmol of Trolox equivalents/g of defatted sample.

2.8. Reducing power

The reducing power (Oyaizu, 2006) of the extracts was determined as described by de Camargo, Regitano-d'Arce, Gallo, and Shahidi (2015). Briefly, 1.0 ml of sample or Trolox standard was mixed with 2.5 ml of a 0.2 M phosphate buffer, and 2.5 ml of a 1% (w/v) solution of potassium ferricyanide was incubated in a water bath at 50 °C for 20 min. Aliquots of a 10% (w/v) trichloroacetic acid (2.5 ml) were added to the mixture and then centrifuged at 1650*g* for 10 min. The upper layer of solution (1 ml) was combined with 2.5 ml of distilled water and 0.5 ml of a 0.1% (w/v) solution of ferric chloride. Only potassium ferricyanide and ferric chloride were prepared in 1 M HCl. Different concentrations (2–10 mM) of Trolox were used to prepare the standard curve. The absorbance of the reaction mixture was read at 700 nm and the results were expressed as μμmol of Trolox per gram of defatted sample.

2.9. Measurement of iron(II) chelation capacity

The capacity of phenolic extracts to chelate ferrous ions was estimated according to the procedure by Dinis, Madeira, and Almeida (1994). In brief, 0.4 ml of extracts was added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by addition of 5 mM ferrozine (0.2 ml), and the total volume was adjusted to 4 ml with distilled water. The mixture was vigorously shaken and left to react at room temperature for 10 min. The absorbance of the reaction mixture was read at 562 nm. Appropriate blanks were prepared with 0.4 ml of the sample and 3.6 ml of distilled water for background subtraction. Different concentrations (0.05–2 mM) of EDTA were used to prepare the standard curve. Iron chelation capacities of the extracts were calculated using the following equation:

$$\text{Fe(II) chelation capacity } (\%) = (1 - \text{Absorbance of sample}) \times 100/\text{absorbance of blank}.$$

The results were expressed as micromoles of EDTA equivalents per gram of defatted sample.

2.10. Effect of berry seed meal extracts on preventing cupric ion induced human low density lipoprotein (LDL) cholesterol peroxidation

To evaluate inhibitory activities of berry seed meal extracts towards human LDL-cholesterol oxidation, the assay of Chandrasekara and Shahidi (2011a) was employed. Human LDL-cholesterol (in PBS, pH 7.4, with 0.01% EDTA) was dialysed against 10 mM PBS (pH 7.4, 0.15 M NaCl) for 12 h, under nitrogen at 4 °C, and EDTA-free LDL was subsequently diluted with PBS to obtain 0.1 mg/ml. The diluted LDL cholesterol solution (0.8 ml) was mixed with 100 μμl of extract (0.125 and 0.5 mg/ml) in an Eppendorf tube. Oxidation of LDL-cholesterol was initiated by adding 0.1 ml of 100 μM $CuSO_4$ solution in distilled water. The mixture was incubated at 37 °C for 22 h. The initial absorbance (time zero) was read at 234 nm immediately after mixing and conjugated diene (CD) hydroperoxides formed at the end of 22 h were measured. The corrected absorbance at 22 h, against time zero, was employed to calculate the percentage inhibition of CD formation using the following equation:

$$\text{Inhibition of CD formation } (\%) = (\text{Abs}_{\text{oxidised}} - \text{Abs}_{\text{sample}}) / (\text{Abs}_{\text{oxidised}} - \text{Abs}_{\text{native}}) \times 100,$$

where $\text{Abs}_{\text{oxidised}}$ = absorbance of LDL mixture and distilled water with $CuSO_4$ only, $\text{Abs}_{\text{sample}}$ = absorbance of LDL with extract and $CuSO_4$, and $\text{Abs}_{\text{native}}$ = absorbance of LDL with distilled water.

2.11. Inhibition of peroxyl and hydroxyl radical induced supercoiled DNA strand scission

The inhibition activity of the berry seed meal extracts against supercoiled DNA strand scission induced by peroxyl radical was determined according to the method of Chandrasekara and Shahidi (2011a). Plasmid supercoiled DNA (pBR 322) was dissolved in 0.5 M PBS pH 7.4. The DNA (5 μl) was added to 5 μl of extract samples, 10 μl of AAPH (7 mM) dissolved in PBS. The mixture was mixed well and incubated at 37 °C for 1 h. At the end of 1 h incubation, the loading dye (consisting 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol) was added to the sample. The gel was prepared in a 40 mM Tris–acetic acid–EDTA buffer, 1 mM EDTA, pH 8.5. After that, 5 μl SYBR Safe were added to DNA gel. The gel was allowed to set for 30 min. Samples (10 μl) were added into wells carefully and the gel electrophoresis was running at 80 Volt for 2 h. DNA strands were visualised under ultraviolet light. The images were acquired with a Sony digital camera under UV light and analysed using AlphaEase stand-alone software (Alpha Innotech Co., San Leandro, CA). The photos of the control and treated DNA strands, visualised under UV light, of representative samples can be visualised (Supplementary material). For hydroxyl radical-induced DNA oxidation, 5 μμl of test compounds, were added into an Eppendorf tube and the solvent was evaporated under a stream of nitrogen. To each tube, the following reagents were then added in the order stated: 5 μl of PBS (pH 7.4), 5 μl of

supercoiled pBR322 DNA, 5 μl of H_2O_2 and 5 μl of $FeSO_4$ were mixed well and incubated at 37 °C for 1 h.

The protective effect of extracts was calculated as DNA retention (%) based on the following equation:

$$\text{DNA retention } (\%) = (\text{Supercoiled DNA content in sample} / \text{supercoiled DNA content in control}) \times 100.$$

2.12. Statistical analysis

The experimental design was randomised with three replications and the results were analysed using Tukey's test ($p < 0.05$) and SPSS statistics 21 for Windows (SPSS Inc., Chicago, IL).

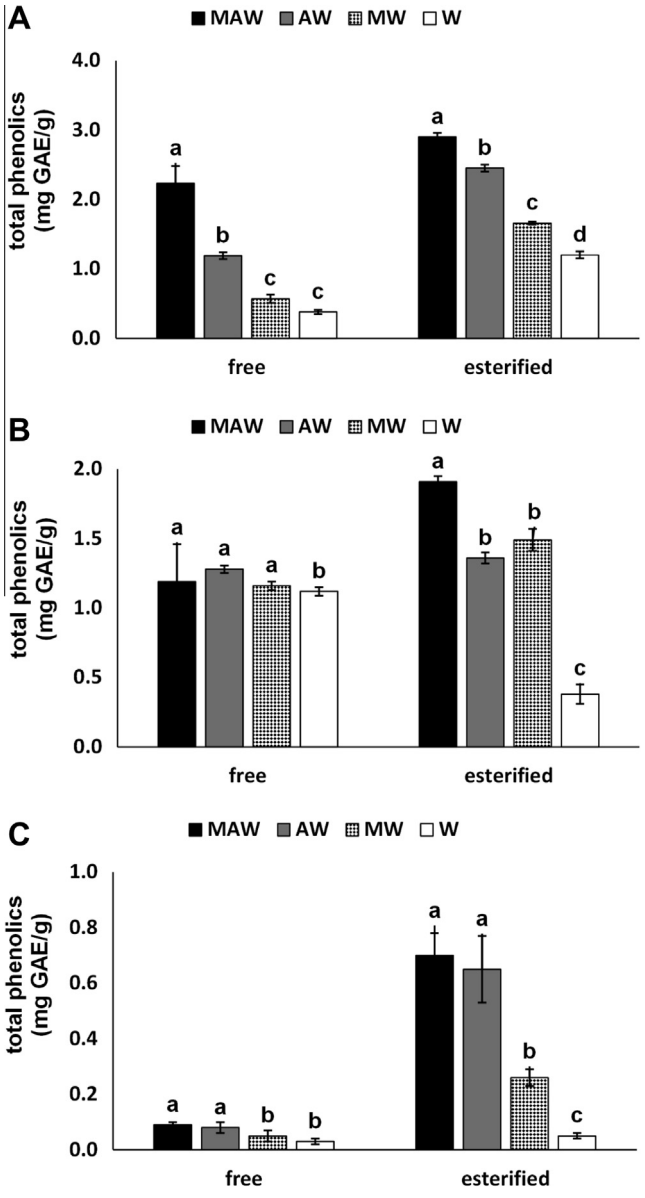

Fig. 1. Effect of solvent type on the extraction of soluble free and esterified phenolics of (A) blackberry, (B) black raspberry, and (C) blueberry seed meals. MAW is methanol–acetone–water (7:7:6, v/v/v); AW is acetone–water (80:20, v/v); MW is methanol–water (70:30, v/v); and W is water. GAE is gallic acid equivalents. DM is dry matter.

3. Results and discussion

3.1. Selection of solvent for the preparation of phenolic extracts

The yield of phenolics extracted differs based on the concentration and type of solvent, which exhibit varying polarities, extraction time, and solid to solvent ratio (de Camargo et al., 2014c). Furthermore, the pH and temperature of the medium also affect the amount of phenolics extracted. However, the solvent and the chemical properties of the sample have the most important influence under the same conditions of time and temperature (de Camargo et al., 2014c). The total phenolics have been found to exhibit a significant correlation with several antioxidant assays, such as ABTS radical cation, DPPH radical, H_2O_2, and hydroxyl radical scavenging capacities, as well as the reducing power, copper-induced LDL-cholesterol oxidation, and supercoiled plasmid DNA strand breakage inhibition (Alshikh, de Camargo, & Shahidi, 2015; Augusto et al., 2014; da Silva et al., 2015; de Camargo, Regitano-d'Arce, Gallo, & Shahidi, 2015; de Camargo et al., 2014a). Thus, in the present study, the selection of solvent for extraction of soluble phenolics was based on the total phenolics. Four different solvents, namely methanol–acetone–water (7:7:6, v/v/v) (Krygier et al., 1982), acetone–water (80:20, v/v) (Alasalvar, Karamać, Amarowicz, & Shahidi, 2006), methanol–water (70:30, v/v) (Naczk, Shahidi, & Sullivan, 1992), and water (de Camargo et al., 2014c) were tested. The evaluation of the fraction containing soluble free and esterified phenolics (Fig. 1) showed that, in general, the highest total phenolic yields were obtained using methanol–acetone–water (7:7:6, v/v/v), followed by acetone–water (80:20, v/v). These two solvent systems were used for identification (Table 2) and quantification (Table 3) of soluble free and esterified phenolics.

3.2. Identification and quantification of phenolic compounds

The extracts of berry seed meals showed great diversity in the phenolics present. The MS spectra of soluble (free and esterified) and insoluble-bound phenolics, positively or tentatively identified,

Table 1
Total phenolics, radical scavenging capacity, reducing power and chelation power of berry seed meals.

	Free	Esterified	Insoluble-bound
Total phenolics (mg GAE/g)			
Blackberry	2.23 ± 0.05Ca	2.90 ± 0.06 Ba	7.93 ± 0.20Aa
Black raspberry	0.80 ± 0.10Cb	1.90 ± 0.15Bb	4.60 ± 0.20 Ab
Blueberry	0.09 ± 0.12Cc	0.70 ± 0.97Bc	1.05 ± 0.12Ac
Hydroxyl radical scavenging activity (μmol TE/g)			
Blackberry	32.6 ± 0.08Ca	38.6 ± 0.85Ba	53.8 ± 0.50Aa
Black raspberry	2.40 ± 0.30Cb	16.7 ± 1.81Bb	48.3 ± 1.20Ab
Blueberry	1.60 ± 0.56Cc	16.8 ± 0.76Bb	18.6 ± 0.10Ac
Oxygen radical absorbance capacity (μmol TE/g)			
Blackberry	6.50 ± 1.10Cb	20.9 ± 0.03Ba	32.8 ± 1.30Aa
Black raspberry	14.0 ± 0.40Ba	16.0 ± 0.20Ab	16.5 ± 0.60Ab
Blueberry	3.25 ± 0.07Cc	6.70 ± 0.30Bc	8.30 ± 0.02Ac
Reducing power (μmol TE/g)			
Blackberry	17.6 ± 0.20Ba	18.0 ± 0.42Ba	52.2 ± 0.60Aa
Black raspberry	6.50 ± 0.03Cb	20.9 ± 1.10Ba	32.8 ± 3.02Ab
Blueberry	2.77 ± 0.13Cc	4.19 ± 0.13Ab	5.00 ± 0.25Ac
Chelation capacity (μmol TE/g)			
Blackberry	63.5 ± 0.20Ca	66.4 ± 0.01Ba	68.6 ± 0.30Aa
Black raspberry	20.5 ± 0.01Cb	22.5 ± 0.02Bb	26.2 ± 0.01Ab
Blueberry	6.50 ± 0.01Cc	8.20 ± 0.01Bc	10.1 ± 0.00Ac

Data represent the mean values for each sample ± standard deviations ($n = 3$). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).

are shown in Table 2. The identification of soluble phenolics was carried out in extracts obtained with methanol–acetone–water (7:7:6, v/v/v) and acetone–water (80:20, v/v), which observed the highest total phenolics (Fig. 1). Further quantification (Table 3) of soluble (free and esterified) phenolics using HPLC showed metha nol–acetone–water (7:7:6, v/v/v) as the best option when compared with acetone–water (80:20, v/v). As an example, metha nol–acetone–water showed, in general, the highest extraction of the major phenolic acids, flavonoids, proanthocyanidins, and anthocyanins. This is evident for gallic acid and its derivative (gallic hexoside), quercetin and its derivatives (quercetin-3-*O*-glucuronide and quercetin pentose), procyanidin dimer B, and peonidin derivatives (peonidin-3-*O*-arabinoside and peonidin-3-*O*-glucoside), respectively. According to Naczk and Shahidi (1989), the concentration of insoluble-bound phenolics was not affected by different solvents used to extract soluble phenolics; thus, in the present study, the fraction containing insoluble-bound phenolics was investigated in the residue from the

Table 2
Phenolic compounds identified in berry seed meals.

		Blackberry	Black raspberry	Blueberry	MW	*m/z*	Other product ions (*m/z*)
Phenolic acids							
1	MAW	F, E, B	F, E, B	F, E, B	Protocatechuic acid*		
	AW	F, E	F, E	F, E	154	153	109
2	MAW	F, E, B	F, E, B	F, E, B	*p*-Coumaric acid*		
	AW	F, E		F, E	164	163	139, 119
3	MAW	F, E, B	F, E, B	F, E, B	Gallic acid*		
	AW	F, E	F, E	F, E	170	169	125
	MAW						
4	AW	F, E, B	F, E, B	F, E, B	Caffeic acid*		
	MAW	F, E	F, E	F	180	179	135
	AW						
5	MAW			F, E, B	Syringic acid		
	AW	E	F, E	F, E	198	197	153
6	MAW	F, E, B	F, E, B	F, E, B	Gallic hexoside		
	AW	F, E	F, E	F, E	332	331	169
Flavonoids							
7	MAW	F, E, B	F, E, B	F, E, B	(+)-Catechin*		
	AW	F, E	F, E	F, E	290	289	245, 205, 179
8	MAW	F, E, B	F, E, B	F, E, B	(−)-Epicatechin*		
	AW	F, E	F, E	F, E	290	289	245, 205, 179
9	MAW	F, E, B	F, E, B	F, E, B	Quercetin*		
	AW	F, E	F, E	F, E	302	301	179, 121
10	MAW	F, E, B	F, E, B	F, E, B	Epigallocatechin		
	AW	F, E	F, E	F, E	306	305	287, 247, 179, 151
11	MAW	B	E	F, E, B	Myricetin		
	AW		F, E	F, E	318	317	179, 151
12	MAW	E, B	F, E	F, E, B	Quercetin pentose		
	AW	F, E	F	F, E	434	433	301
13	MAW	F, E, B	F, E, B		Epicatechin gallate		
	AW	F	F, E		442	441	289, 169
14	MAW			F, E, B	Kaempferol hexoside		
	AW				448	447	
15	MAW	F, E, B	F, E, B		Quercetin-3-*O*-glucuronide		
	AW	F, E	F, E		478	477	301
Procyanidins							
16	MAW	F, E, B	F, E, B	F, E, B	Procyanidin dimer B1		
	AW	F, E	F, E	F, E	578	577	451, 425, 289
17	MAW	F, E	F, E, B		Procyanidin dimer B2		
	AW	F, E	E		578	577	451, 425, 289
18	MAW	F, E, B	F, E, B	F, E, B	Procyanidin dimer B3		
	AW	F, E	F, E	F, E	578	577	451, 425, 289
19	MAW	F, E, B	F, E, B		Procyanidin dimer B4		
	AW	E			578	577	451, 425, 289
Anthocyanins							
20	MAW			F, B	Peonidin-3-*O*-arabinoside		
	AW				434	433	301
21	MAW			F, E, B	Petunidin-3-*O*-arabinoside		
	AW			F, E	450	449	317
22	MAW	F, E, B	F, E	F, E, B	Peonidin-3-*O*-glucoside		
	AW	F, E	F, E	F, E	464	463	301
23	MAW			B	Delphinidin-3-*O*-hexoside		
	AW				466	465	303
24	MAW			F, E, B	Petunidin-3-*O*-galactoside		
	AW			F, E	480	479	317
25	MAW			F, B	Cyanidin-3-(6-acetyl)glucoside		
	AW				492	491	287

MAW is methanol–acetone–water (7:7:6, v/v/v); AW is acetone–water (80:20, v/v). F, E, and B are free, esterified and insoluble-bound phenolics, respectively. Identification of insoluble-bound phenolics was carried out only in the extracts obtained with MAW, which rendered the highest extraction of soluble phenolics and were selected for the remaining experiments. For details, see Section 3.1.

* Compounds tentatively identified using authentic standards.

Table 3
The contents of phenolic compounds (μg/100 g of defatted sample) of berry seed meals.

	Blackberry	Black raspberry	Blueberry	Blackberry	Black raspberry	Blueberry
Phenolic acids						
Protocatechuic acid						
Free	48.5 ± 0.1Ac[1]	10.4 ± 0.9Cc[1]	37.9 ± 2.1Ba[1]	14.9 ± 1.1Ab[2]	9.22 ± 0.7Bb[1]	8.01 ± 1.4Bb[2]
Esterified	133 ± 1.4Ab[1]	44.0 ± 3.0Bb[1]	19.4 ± 1.6Cb[2]	39.9 ± 1.4Aa[2]	13.1 ± 1.0Ba[2]	34.0 ± 2.1Aa[1]
Insoluble-bound	221 ± 0.1Aa	93.5 ± 1.9Ba	39.7 ± 1.1Ca	*	*	*
p-Coumaric acid						
Free	37.8 ± 1.79Aa	26.8 ± 1.3Ac	26.0 ± 4.4Ab[1]	tr	nd	29.9 ± 0.9a[1]
Esterified	26.9 ± 0.62Cb	484 ± 1.7Aa	57.4 ± 0.9Ba[1]	tr	nd	30.0 ± 1.1a[2]
Insoluble-bound	25.5 ± 0.59Bb	243 ± 1.5Bb	23.8 ± 0.2Ab	*	*	*
Gallic acid						
Free	53.2 ± 0.1Cc[1]	345 ± 1.4Aa[1]	337 ± 2.3Bb[1]	27.1 ± 0.8Ab[2]	13.5 ± 1.1Bb[2]	6.11 ± 1.2Ca[1]
Esterified	242 ± 0.2Ab[1]	53.2 ± 0.8Cc[2]	79.3 ± 1.1Ac[1]	235 ± 1.1Aa[2]	89.7 ± 1.1Ba[1]	7.10 ± 1.5Ca[2]
Insoluble-bound	345 ± 1.4Ba	242 ± 1.3Cb	356 ± 1.4Aa	*	*	*
Caffeic acid						
Free	52.0 ± 0.3Ac[1]	6.30 ± 1.1Bc[2]	51.9 ± 1.5Aa[1]	20.3 ± 1.8Aa[2]	20.3 ± 1.0Ab[1]	20.3 ± 2.5Ab[2]
Esterified	69.6 ± 0.3Bb[1]	114 ± 1.3Ab[1]	27.0 ± 2.9Cb[2]	27.0 ± 2.0Ba[2]	25.4 ± 1.3Ba[2]	44.8 ± 0.6Aa[1]
Insoluble-bound	217 ± 1.5Aa	158 ± 1.8Ba	25.4 ± 0.6Cb	*	*	*
Syringic acid						
Free	nd	nd	91.0 ± 0.2b[1]	nd	30.0 ± 1.4A	26.0 ± 1.10Aa[2]
Esterified	nd	nd	40.8 ± 0.3c[1]	279 ± 1.6A	tr	27.3 ± 0.36Aa[2]
Insoluble-bound	nd	nd	93.6 ± 0.6a	*	*	*
Gallic hexoside						
Free	37.2 ± 0.2Cc[1]	161 ± 0.6Aa[1]	46.3 ± 0.6Bb[1]	29.6 ± 0.4Ab[2]	26.3 ± 1.7Ab[2]	13.3 ± 0.4Ba[2]
Esterified	268 ± 0.1Ab[2]	26.8 ± 0.3Bc[1]	14.7 ± 1.5Cc[1]	388 ± 1.5Aa[1]	131 ± 1.8Ba[2]	4.61 ± 0.7Cb[1]
Insoluble-bound	703 ± 0.4Aa	53.2 ± 0.8Bb	103 ± 1.0Ca	*	*	*
Flavonoids						
Catechin						
Free	6.16 ± 0.1Bc[1]	6.30 ± 1.1Bc[1]	65.9 ± 5.8Aa[1]	6.91 ± 0.4ABb[1]	8.71 ± 1.2Aa[1]	3.80 ± 0.4Ba[2]
Esterified	124 ± 0.1Bb[1]	238 ± 1.6Aa[1]	12.9 ± 0.9Cb[1]	63.8 ± 1.4Aa[2]	4.31 ± 0.9Bb[2]	4.31 ± 0.4Ba[2]
Insoluble-bound	102 ± 0.1Aa	40.0 ± 2.9Cb	76.6 ± 0.6Ba	*	*	*
Epicatechin						
Free	137 ± 0.8Ab[1]	6.31 ± 0.8Cc[1]	17.0 ± 1.7Ba[1]	30.6 ± 2.6Bb[2]	71.9 ± 1.30A[2]	5.61 ± 0.8Ca[2]
Esterified	326 ± 1.6Aa[1]	67.5 ± 0.8Bb	11.3 ± 0.4Cb[1]	162 ± 2.8Aa[2]	tr	4.25 ± 1.1Ba[2]
Insoluble-bound	116 ± 0.8Bc	311 ± 2.8Aa	7.80 ± 0.6Cb	*	*	*
Quercetin						
Free	70.8 ± 0.9Ac[1]	69.1 ± 1.4Ac[1]	26.0 ± 1.4Ba[1]	49.4 ± 0.9Ab[2]	14.4 ± 0.9Bb[2]	12.7 ± 0.5Ba[2]
Esterified	233 ± 0.3Aa[1]	160 ± 1.4Bb[1]	9.11 ± 0.5Cb[2]	54.9 ± 1.4Ba[2]	97.5 ± 1.4Aa[2]	11.0 ± 0.7Ca[1]
Insoluble-bound	207 ± 0.3Bb	326 ± 2.4Aa	27.4 ± 0.6Ca	*	*	*
Epigallocatechin						
Free	78.0 ± 1.4Ab[1]	22.0 ± 1.4Bc[1]	tr	22.4 ± 0.7Aa[2]	15.3 ± 4.0Aa[1]	5.20 ± 0.3Ba
Esterified	138 ± 1.0Aa[1]	86.5 ± 0.9Bb[1]	tr	24.5 ± 0.7Aa[2]	22.6 ± 1.0Aa[2]	4.60 ± 0.7Ba
Insoluble-bound	tr	136 ± 2.2Aa	40.4 ± 1.0B	*	*	*
Myricetin						
Free	nd	nd	51.0 ± 0.3a[1]	nd	28.9 ± 1.4Ab	13.5 ± 1.0Ba[1]
Esterified	209 ± 2.1A	177 ± 1.4B[2]	27.9 ± 0.3Cb[1]	nd	199 ± 1.8Aa[1]	12.8 ± 0.4Ba[2]
Insoluble-bound	nd	nd	26.3 ± 0.2c	*	*	*
Quercetin pentose						
Free	nd	32.0 ± 3.4Ab[2]	18.0 ± 1.6Bc[1]	46.9 ± 1.0Bb	54.6 ± 0.9A[1]	12.2 ± 0.2Ca[2]
Esterified	144 ± 2.8Bb[2]	231 ± 1.6Aa	45.7 ± 0.5Cb[1]	207 ± 2.5Aa[1]	nd	9.81 ± 1.6Ba[2]
Insoluble-bound	330 ± 0.6Aa	nd	57.5 ± 0.6Ba	*	*	*
Epicatechin gallate						
Free	208 ± 0.48Ab[1]	40.0 ± 0.7Bc[2]	nd	77.7 ± 1.8A[2]	51.8 ± 1.2Ba[1]	nd
Esterified	861 ± 1.20Aa	295 ± 2.6Ba[1]	nd	nd	13.5 ± 0.7b[2]	nd
Insoluble-bound	117 ± 1.68Ac	93.7 ± 1.7Bb	nd	*	*	*
Kaempferol hexoside						
Free	nd	nd	90.7 ± 0.3a	nd	nd	nd
Esterified	nd	nd	24.3 ± 0.4c	nd	nd	nd
Insoluble-bound	nd	nd	68.4 ± 2.0b	*	*	*
Quercetin 3-O-glucoronide						
Free	792 ± 2.0Aa[1]	219 ± 3.3Bc[1]	nd	508 ± 2.8Aa[2]	195 ± 5.7Ba[2]	nd
Esterified	647 ± 0.5Bb[1]	714 ± 2.3Ab[1]	nd	237 ± 0.9Ab[2]	68.1 ± 1.4Bb[2]	nd
Insoluble-bound	513 ± 1.4Bc	1149 ± 11Aa	nd	*	*	*
Procyanidins						
Procyanidin dimer B1						
Free	38.0 ± 0.0Ac[1]	6.60 ± 1.9Cb[1]	14.3 ± 1.5Bb[1]	7.18 ± 1.1Ab[2]	10.3 ± 1.1Aa[1]	10.5 ± 0.8Aa[1]
Esterified	40.5 ± 0.7Ab[1]	41.6 ± 1.9Aa[1]	21.9 ± 1.4Ba[1]	19.9 ± 1.3Aa[2]	5.14 ± 0.3Bb[2]	6.07 ± 0.1Bb[2]
Insoluble-bound	351 ± 0.0Aa	36.0 ± 3.5Ba	24.3 ± 0.9Ca	*	*	*

Table 3 (*continued*)

	Blackberry	Black raspberry	Blueberry	Blackberry	Black raspberry	Blueberry
Procyanidin dimer B2						
Free	96.8 ± 0.4Aa[1]	28.6 ± 2.0Bc	nd	25.5 ± 0.8b[2]	nd	nd
Esterified	65.3 ± 0.2Bb[1]	109 ± 0.9Aa[1]	nd	70.6 ± 1.6Aa[2]	7.01 ± 1.5B[2]	nd
Insoluble-bound	nd	97.5 ± 1.9b	nd	*	*	*
Procyanidin dimer B3						
Free	27.8 ± 0.4Ab[1]	22.0 ± 1.7Bb[1]	29.9 ± 0.2Ab[1]	6.20 ± 0.5Ab[2]	4.21 ± 0.5Ab[2]	4.71 ± 1.0Aa[2]
Esterified	19.3 ± 0.4Bc[2]	63.0 ± 4.4Aa[1]	6.50 ± 0.5Cc[1]	26.8 ± 1.6Aa[1]	8.31 ± 1.0Ba[2]	3.61 ± 0.7Ba[2]
Insoluble-bound	102 ± 0.5Aa	71.4 ± 1.8Ba	45.7 ± 0.5Ca	*	*	*
Procyanidin dimer B4						
Free	20.6 ± 0.6Ac	7.40 ± 0.9Bc	nd	nd	nd	nd
Esterified	43.4 ± 0.5Ba[1]	96.7 ± 0.7Aa	nd	25.8 ± 3.7[2]	nd	nd
Insoluble-bound	31.0 ± 0.1Bb	46.6 ± 0.7Ab	nd	*	*	*
Anthocyanins						
Peonidin-3-O-arabinoside						
Free	nd	nd	116 ± 4.9	nd	nd	nd
Esterified	nd	nd	nd	nd	nd	nd
Insoluble-bound	nd	nd	tr	*	*	*
Petunidin-3-O-arabinoside						
Free	nd	nd	93.7 ± 1.4[1]	nd	nd	9.09 ± 0.4[2]
Esterified	nd	nd	tr	nd	nd	tr
Insoluble-bound	nd	nd	tr	*	*	*
Peonidin-3-O-glucoside						
Free	244 ± 1.4A[1]	47.8 ± 1.3C[2]	57.8 ± 0.6B[1]	129 ± 2.0A[2]	55.8 ± 1.4B[1]	15.7 ± 0.8C[2]
Esterified	tr	tr	tr	tr	tr	tr
Insoluble-bound	tr	nd	tr	*	*	*
Delphinidin-3-O-hexoside						
Free	nd	nd	23.1 ± 1.3	nd	nd	nd
Esterified	nd	nd	nd	nd	nd	nd
Insoluble-bound	nd	nd	nd	*	*	*
Petunidin-3-O-galactoside						
Free	nd	nd	44.0 ± 2.8[1]	nd	nd	12.9 ± 1.3[2]
Esterified	nd	nd	tr	nd	nd	tr
Insoluble-bound	nd	nd	tr	*	*	*
Cyanidin-3-(6-acetyl)galactoside						
Free	nd	nd	75.2 ± 1.4a	nd	nd	nd
Esterified	nd	nd	nd	nd	nd	nd
Insoluble-bound	nd	nd	tr	*	*	*

Data represent mean values for each sample ± standard deviations ($n = 3$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$). Means followed by the same capital letters in a row are not significantly different ($p > 0.05$) within the same solvent (methanol–acetone–water or acetone–water). Means followed by the same superscript figures in a row are not significantly different ($p > 0.05$) within the same sample but using different solvent (methanol–acetone–water vs acetone–water). nd is not detected and tr is trace.

* Insoluble-bound phenolics were not determined after extraction of soluble phenolics with acetone–water.

extraction using methanol–acetone–water (7:7:6, v/v/v), which exhibited the highest extraction of soluble phenolics. Phenolic acids and flavonoids were investigated using negative ion detection and anthocyanins were detected using positive ion detection.

Five phenolic acids were identified (compounds **1**–**5**) in berry seed meals: protocatechuic acid, *p*-coumaric acid, gallic acid, caffeic acid, and syringic acid. They were identified by comparison of their ion fragmentation patterns and retention times as those of authentic standards. Gallic hexoside (compound **6**), also known as monogalloyl glucose, gave deprotonated ions at *m*/*z* 331 and 169 in MS and MS^2, respectively, which is accordance with a previous study (de Camargo et al., 2014a). Protocatechuic acid, *p*-coumaric acid, gallic acid, and caffeic acid were also detected in blueberries and blackberries (Zadernowski, Naczk, & Nesterowicz, 2005). The same authors also reported the presence of syringic acid in blueberries, but not in blackberries.

The monomeric of flavan-3-ols (+)-catechin, and (−)epicatechin (compounds **7** and **8**, respectively) gave the same deprotonated molecular ion $[M-H]^-$, exhibiting a *m*/*z* at 289, and MS^2 at 245, both showing loss of CO_2 $[M-H-44]^-$ (de Camargo, Regitano-d'Arce, Gallo, & Shahidi, 2015), however, their comparison with authentic standards, which eluted in different retention times, enabled their positive identification. Quercetin and myricetin (compounds **9** and **11**, respectively) were positively identified based on retention time and mass spectral characteristics of their corresponding standards. (+)-Catechin, (−)epicatechin, quercetin and myricetin were previously reported in Chilean wild blackberry (Céspedes et al., 2010). Compound **10**, with a deprotonated ion at *m*/*z* 305, showed the same fragmentation pattern of a previous work (Chandrasekara & Shahidi, 2011b) in MS^2, thus being tentatively identified as epigallocatechin. The presence of epigallocatechin in blueberries was previously reported (Huang, Zhang, Liu, & Li, 2012). Quercetin pentose (*m*/*z* 433 → *m*/*z* 301) was tentatively identified as compound **12** (Chandrasekara & Shahidi, 2011b). Epicatechin gallate (compound **13**), which is a galloylated flavan-3-ol, was tentatively identified due to its deprotonated molecular ion at *m*/*z* 441, followed by *m*/*z* at 289 in MS^2 and at *m*/*z* 169, the last one indicating the presence of a gallic acid attached (de Camargo et al., 2014a). Compound **14** showed loss of hexose $[M-H-162]^-$ and a gave product ion at *m*/*z* 285 (kaempferol) indicating its identity as kaempferol hexoside (de Camargo et al., 2014a). Kaempferol and its derivative have already been reported in blueberries (Zheng & Wang, 2003). Compound **15** was tentatively identified as quercetin 3-*O*-glucuronide (de Camargo et al., 2014a), as it gave a deprotonated ion at *m*/*z* 477 and MS^2 at 301. Quercetin 3-*O*-glucuronide has been reported in blackberries (Mertz, Cheynier, Günata, & Brat, 2007). Procyanidin dimers B1 to B4 were tentatively identified according to previous studies (de Camargo et al.,

2014a, 2015) and have also been found in Chilean wild blackberries (Céspedes et al., 2010). These peaks had the same ionisation pattern, which is common for proanthocyanidins isomers; however, accurate stereoisomer identification requires nuclear magnetic resonance (NMR) analysis. The peaks were named B1 to B4 according to their different elution time.

Compounds **20** and **21** were tentatively identified as peonidin-3-*O*-arabinoside (Seeram, Adams, Hardy, & Heber, 2004) and petunidin-3-*O*-arabinoside (Wu & Prior, 2005), due to their molecular ions $[M-H]^+$ obtained by MS at *m*/*z* 433 and 449, respectively. Their respective *m*/*z* signals in MS^2 were at 301 and 317, indicating loss of arabinose $[M-H-132]^+$. Peonidin-3-*O*-arabinoside was also found in blueberries (Kalt et al., 2008). These authors also reported the presence of such compound in the liver, eyes, cortex and cerebellum of pigs fed with blueberries. Petunidin-3-*O*-arabinoside has also been detected by Zheng and Wang (2003) in blueberries. Compound **23** could be delphinidin-3-*O*-galactoside or delphinidin-3-*O*-glucoside as both have been previously identified in blueberries (Wu & Prior, 2005) thus, in the present study, the compound was tentatively identified as delphinidin-3-*O*-hexoside due to its pseudomolecular ion at *m*/*z* 465 and 303 in MS and MS^2, respectively. Petunidin-3-*O*-galactoside, which gave a *m*/*z* at 479 and 317 in MS and MS^2 was tentatively identified as compound **24**. This anthocyanin have already been identified in blueberries (Wu & Prior, 2005). Compound **25** was tentatively identified as cyaniding-3-(6-acetyl)glucoside according to the same fragmentation pattern previously reported (Wu & Prior, 2005), which gave molecular ions $[M-H]^+$ at *m*/*z* 491 and 287 in MS and MS^2, respectively. The same authors reported the presence of such a compound in blueberries.

In general, gallic acid and its derivative (gallic hexoside) gave the major phenolic acid contribution in berry seed meals (Table 3). Black raspberry seed meals contained the highest level of phenolic acids in both solvent systems and blueberry seed meals contained the lowest. The phenolic profile of different berries has extensively been discussed; however, due to different type of sample used (fruits vs seed meals), comparing the amounts found in the present study with literature data is not possible. Additionally, most studies have been conducted in crude phenolic extracts, comprising soluble free plus esterified phenolics, which is different in the present study, in which the quantification was carried out in different fractions (free, esterified, and insoluble-bound phenolics). Furthermore, climatic and stress conditions, as well as soil quality, play important roles in the content of phenolics (de Camargo et al., 2014a).

Phenolic acids liberated from the insoluble-bound fraction were the predominant phenolic acids in the berry seed meals (Table 3). This fraction comprised from 38% (black raspberry seed meals) to 61% (blackberry seed meals) of the total phenolic acids present in the berry seed meals. The comparative analyses of the phenolic acids in the three phenolic fractions have revealed lower amounts of free phenolic acids compared with the other two fractions. It has been reported that phenolic acids in their free forms are very rarely present in plants, whereas the majority of phenolic acids are present in their insoluble-bound forms (de Camargo et al., 2014a; Zuo, Wang, & Zhan, 2002). Protocatechuic acid, *p*-coumaric acid, gallic acid, caffeic acid, and gallic hexoside were present in all forms (free, esterified, or insoluble-bound) of all samples in the present study; however, syringic acid was detected only in blueberry seed meals.

Thirteen flavonoids were found in the different fractions of analysed berry seed meals, showing different concentrations and distribution within these fractions. (+)-Catechin, (−)-epicatechin, quercetin, epigallocatechin, procyanidin dimer B1, and procyanidin dimer B3 were identified in all fractions from all samples. Quercetin and its two derivatives (quercetin-3-*O*-glucuronide and quercetin pentose) were detected in berry seed meals and provided contributions of 57% and 61% of total flavonoids in blackberry and black raspberry, respectively, thus showing a major contribution. Other authors (Gavrilova, Kajdžanoska, Gjamovski, & Stefova, 2011) also indicated that quercetin derivatives are the dominant flavonols in blueberries. In the present study, quercetin-3-*O*-glucuronide was not detected in blueberry seed meals, which explains the fact that quercetin and its two derivatives were not dominant flavonoids in blueberry seed meals. Procyanidins (B1–B4) gave the highest contribution of total flavonoids in blueberries and were the second most abundant compounds in blackberry and black raspberry. Kaempferol hexoside, which was the second most abundant flavonoid in blueberries, was not found in blackberry and black raspberry seed meals.

Berries are rich in anthocyanins, compounds that provide pigmentation to fruits and serve as natural antioxidants. However, the present study shows that the amount of anthocyanins in berry seed meals is very minimum. This is because the extraction system employed for the fraction containing soluble esterified and insoluble-bound phenolics would probably degrade any anthocyanin present to phenolic acids, due to the changes in the pH during the process. Thus, the only anthocyanin identified in the blackberry and black raspberry seed meals was peonidin-3-*O*-glucoside. However, the blueberry seed meals contain a variety of anthocyanins compared with blackberry and black raspberry seed meals. Of the common fruits and vegetables, blueberries are considered to possess the highest content of anthocyanidins (Juranic & Zizak, 2005). However, several factors, such as climate and stress conditions, as well as soil quality, may play important roles in the content of phenolics (de Camargo et al., 2015). Anthocyanins are labile in nature and susceptible to deterioration during processing and storage (da Silva et al., 2014). Huang et al. (2012) also detected higher levels of anthocyanidins in blueberries than in blackberries. In the present study, peonidin derivatives (peonidin-3-O-arabinoside and peonidin-3-O-glucoside) were the major anthocyanins in all berry seed meals.

3.3. Estimation of total phenolic contents

The contents of total phenolics in blackberry, black raspberry, and blueberry seed meals were different, with blackberry seed meals being a richer source of total phenolics than black raspberry seed meals and blueberry seed meals (Table 3). This result is in general agreement with earlier findings (De Souza et al., 2014). These authors reported that the highest amount of total phenolics was present in blackberries, when compared with blueberries. Furthermore, these authors also found higher total phenolics in blackberries as compared with red raspberry, strawberry, and cherry, supporting the data from the present study and indicating blackberries as an important source of phenolics. The amount of total phenolics in the present study lends support to the results obtained using HPLC, where the insoluble-bound fraction contained the highest total phenolics, followed by esterified and free ones. These results agree with the findings of other studies. For example, de Camargo et al. (2014a) reported a high contribution of the insoluble-bound form to the total phenolic content of grape byproducts. In our study, the phenolic contents in the berry seed meals varied greatly and ranged from 0.09 to 7.90 mg GAE/g of defatted meal, in the fractions containing free phenolics from blueberry and those released from their insoluble-bound form of blackberry seed meals, respectively.

3.4. Activity of phenolic extracts from berry seed meals against ROS

Hydrogen peroxide in the presence of ferric ions can produce the hydroxyl radical via the Fenton reaction (de Camargo,

Regitano-d'Arce, Gallo, & Shahidi, 2015). This highly reactive species may cause damage to molecules, such as DNA, proteins and polyunsaturated fatty acids (PUFA) in the membranes (Alshikh, de Camargo, & Shahidi, 2015). Therefore, examination of the hydroxyl radical scavenging activity of the berry seed meal extracts is significant. The results of the present study are in agreement with those reported by other researchers, who have shown that phenolics from plants, or their fractions, present direct scavenging activities against hydroxyl radicals (de Camargo, Regitano-d'Arce, Gallo, & Shahidi, 2015; Wettasinghe & Shahidi, 1999). If berry seeds meal extracts act as an effective antioxidant, there is likely some constituent within the extract that can be employed as hydrogen atom donors and oxygen scavengers. It has been suggested that flavonoids, such as procyanidins, are hydroxyl radical scavengers (de Camargo, Regitano-d'Arce, Gallo, & Shahidi, 2015). In the present study, the extracts exerted significant scavenging effects on hydroxyl radicals and that these effects increased with an increase in the concentration.

The ORAC assay measures the capacity of phenolics to scavenge the peroxyl radicals that characterise this method (Prior, Wu, & Schaich, 2005). The results (Table 3) showed a higher antioxidant capacity of the insoluble-bound phenolics in blackberry seed meals compared with those of the free and esterified fractions. Blueberry seed meals had lower ORAC values, whereas black raspberry seed meals had intermediate ones. Peroxyl radicals are reactive oxygen species, which are related to oxidative processes in both *in vitro* and *in vivo*. They also participate in cell damage, cancer development, inflammation, and heart disease. The demonstrated antiradical capacity of berry seed meals found here may be an indicator of their potential in preventing oxidative related diseases.

3.5. Activity of phenolic extracts from berry seed meals against metal ions

The assay for the determination of reducing power follows the reduction of the ferric form to the ferrous form. As a result of the effect of the phenolic extracts of berry seed meals as electron donors, the radical chain reactions are terminated by converting free radicals to more stable products; hence, these extracts primarily exhibited considerable reducing power. The blackberry seed meal samples had a higher reducing power compared with the black raspberry seed meals and blueberry seed meals (Table 3). Therefore, the phenolics found in blackberry seed meals extracts are powerful electron donors and can terminate the radical chain reactions that may otherwise be very damaging. Although differences in the total phenolics have been found between the fractions containing free and esterified phenolics from blackberry seed meals, as well as between the fractions containing esterified phenolics and insoluble-bound phenolics from blueberry seed meals, no difference was found in their respective reducing power. This highlights the importance of evaluating the activity of phenolic compounds using different methods as they are managed by different mechanisms. However, it is evident the reducing power of all samples tested, which occurs at different extents.

Transition metal ions, which may be introduced by processing methods, can contaminate foods. Bivalent ferrous ions play an important role as catalysts of oxidative processes, leading to the formation of superoxide anion radicals and hydroxyl radicals, via Fenton reactions (Ebrahimzadeh, Nabavi, Nabavi, & Eslami, 2009). Phenolics should be able to form complexes with metals ions and thereby stabilise them. Therefore, they should prevent metal-catalysed initiation and hydroperoxide decomposition reactions. The chelation activities of the phenolic extracts from berry seed meals were examined against Fe(II) and reported as EDTA equivalents. The Fe(II) chelation capacities of the free, esterified, and insoluble-bound phenolics of berry seed meals were in the same trend as observed for the evaluation of total phenolics. However, no difference was found amongst different fractions for all samples. The Haber–Weiss cycle (also known as Fenton reaction) stands by itself. As a cycle, several reactions occur, at which peroxyl and hydroxyl radicals, as well as ferric and ferrous ions, participate. The present study evaluated the potential of berry seed meals against all mentioned compounds and demonstrated in specific methods the efficacy of phenolics extracted from all samples and fractions. These results are good predictors of actual health benefits, which will be also discussed in the following sections, using biological model systems.

3.6. Inhibition of oxidation of human LDL-cholesterol

High levels of oxidised LDL-cholesterol are a risk factor atherosclerosis. In light of this, dietary antioxidants are important in the prevention of atherosclerosis and associated cardiovascular diseases, due to their ability to inhibit LDL-cholesterol oxidation (Chandrasekara & Shahidi, 2011a; de Camargo et al., 2014a). The inhibition effects of berry seed meals at a concentration of 50 ppm against copper-induced human LDL-cholesterol oxidation is shown in Table 4. Because LDL-cholesterol molecules possess antioxidant compounds, such as tocopherol, β-carotene and

Table 4
Effect of phenolic extracts from berry seed meals in preventing cupric ion-induced human low density lipoprotein (LDL) peroxidation (%).

Sample	Free	Esterified	Insoluble-bound	Catechin 25 ppm	Catechin 50 ppm
0 h					
Blackberry	24.82 ± 0.04Da	21.21 ± 0.08Ea	26.31 ± 0.08Ca	34.51 ± 0.07B	39.95 ± 0.05A
Raspberry	18.02 ± 0.07Eb	19.52 ± 0.06Db	19.81 ± 0.06Cb	34.51 ± 0.07B	39.95 ± 0.05A
Blueberry	17.54 ± 0.25Dc	6.46 ± 0.11Ec	18.53 ± 0.34Cc	34.51 ± 0.07B	39.95 ± 0.05A
12 h					
Blackberry	31.30 ± 0.20Ea	35.76 ± 0.12Da	37.41 ± 0.07Ca	42.34 ± 0.16B	48.27 ± 0.08A
Raspberry	23.03 ± 0.07Eb	26.72 ± 0.28Db	29.06 ± 0.12Cb	42.34 ± 0.16B	48.27 ± 0.08A
Blueberry	20.45 ± 0.14Ec	22.81 ± 0.18Dc	24.02 ± 0.03Cc	42.34 ± 0.16B	48.27 ± 0.08A
16 h					
Blackberry	42.71 ± 0.08Ea	47.22 ± 0.03Da	48.71 ± 0.17Ca	57.11 ± 0.03B	63.83 ± 0.04A
Raspberry	42.53 ± 0.35Da	45.05 ± 0.03Cb	42.30 ± 0.09Db	57.11 ± 0.03B	63.83 ± 0.04A
Blueberry	27.06 ± 0.22Eb	31.00 ± 0.02Dc	37.82 ± 0.17Cc	57.11 ± 0.03B	63.83 ± 0.04A
22 h					
Blackberry	50.40 ± 0.05Ea	58.93 ± 0.06Ca	59.93 ± 0.25Da	65.83 ± 0.15B	74.17 ± 0.08A
Raspberry	52.02 ± 0.05Eb	55.23 ± 0.09Db	56.70 ± 0.21Cb	65.83 ± 0.15B	74.17 ± 0.08A
Blueberry	28.04 ± 0.18Ec	33.80 ± 0.07Dc	48.51 ± 0.15Cc	65.83 ± 0.15B	74.17 ± 0.08A

Data represent the mean values for each sample ± standard deviations ($n = 3$). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).

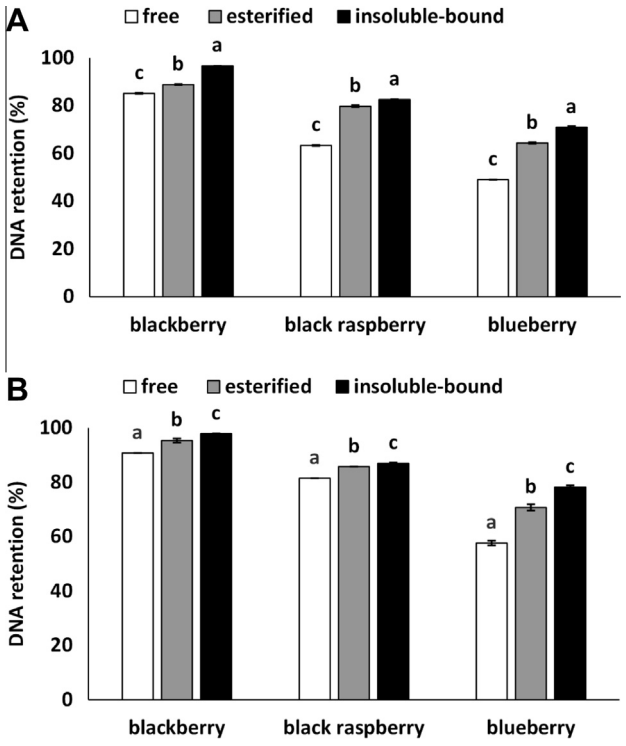

Fig. 2. Supercoiled plasmid DNA strand breakage inhibition of free, esterified, and insoluble-bound phenolics from berry seed meals against (A) hydroxyl and (B) peroxyl radicals. Data represent the mean ± standard deviation of each sample (n = 3). Means with different letters indicate significant differences ($p < 0.05$) amongst fractions within each berry seed meal.

lycopene, the rate of conjugated diene formation was slow at the beginning. For example, phenolics released from their insoluble-bound form from blackberry, which showed the highest inhibition effect, started from 26% at time zero and reached 60% in the evaluation at 22 h. As soon as the depletion of the internal antioxidants of LDL-cholesterol molecules occurs, rapid oxidation is initiated, and this was observed for all samples and fractions as well as catechin, which was used as a positive control. Phenolic compounds from berry seed meals clearly prevented the oxidation of LDL-cholesterol. Therefore, the present results suggest that, in the human body, the inhibition of LDL-cholesterol oxidation may be dependent not only on antioxidants, such as tocopherol, which is found in high concentration in oilseeds (de Camargo et al., 2012), but also on other phenolic compounds present in the test samples. The capacity to eliminate cupric ions from the medium may be the underlying reason for the ability of phenolic compounds to prevent copper ion-mediated LDL-cholesterol oxidation.

The present results showed that berry seed meals were quite effective in reducing the oxidation of human LDL-cholesterol. Depending on the berry seed meals used, and the types of the phenolics (free, esterified, and insoluble-bound), the effects were somewhat different. The preventive effect of phenolic compounds against the oxidation of LDL-cholesterol may be due to their free radical scavenging activity or metal ion chelation properties. Furthermore, phenolic compounds can protect endogenous antioxidants, such as tocopherols, β-carotene, lycopene and ubiquinol, in the LDL-cholesterol molecule or inhibit enzymes, such as xanthine oxidase, involved in the initiation of oxidation or cell-mediated LDL cholesterol oxidation (Chandrasekara & Shahidi, 2011a). The results of this study demonstrate that the phenolic extracts of berry seed meals minimise the metal-stimulated oxidation of LDL-cholesterol.

3.7. Inhibition of peroxyl and hydroxyl radical induced supercoiled DNA strand scission

The breakage of supercoiled plasmid DNA can be induced by ROS, such as hydroxyl and peroxyl radicals. All berry seed meal extracts studied here showed a strong ability to prevent both hydroxyl and peroxyl radical-induced DNA damage in a concentration-dependent manner (Fig. 2A and B). The present results show that a higher level of retention of supercoiled DNA was obtained due to the protection provided by increased concentrations of phenolics from berry seed meals. The protection percentage provided by the extracts against ROS ranged from 49% to 98% at an extract concentration of 0.10 mg/ml. At this concentration, the blackberry seed meals exhibited the highest inhibition against DNA scission (85–97%), whereas the black raspberry seed meals exhibited intermediate inhibition (63–95%), and the blueberry seed meals showed the lowest inhibition (49–78%). Marked scission of supercoiled DNA was caused by the presence of peroxyl radicals, which are more stable than other oxygen radicals and have the capacity to diffuse relatively far from the site of their formation before they react with a target molecule. In the absence of any antioxidant, the peroxyl radical abstracts a hydrogen atom from the nearby DNA to generate new radicals, which results in the induction of a free radical chain reaction leading to the breakage of DNA molecules. Phenolics from berry seed meals were effective in suppressing the hydroxyl radical-induced DNA damage, but the level of protection was lower compared with that against peroxyl radicals. This lends supports to the results found for the antioxidative effect of berry seed meals against peroxyl and hydroxyl radicals (Table 1). Peroxyl radicals have a longer half-life as compared with hydroxyl radicals. The kinetic of reaction is beyond the mandate of the present study; however, it is reasonable to suggest that, due to complex stereochemical structures, hydroxyl groups from phenolics detected in the present study might be not readily available for reaction and would require more time to react with hydroxyl radicals, which, although very toxic, depletes quickly.

The first potential mechanism for the inhibition of supercoiled DNA scission induced by hydroxyl radicals involves the chelation of ferrous ions. The chelation of ferrous ions is required to initiate and catalyse the decomposition of H_2O_2, or the scavenging of H_2O_2, itself and therefore inhibit the production of hydroxyl radicals. The second mechanism is associated with the ability of phenolic extracts to scavenge hydroxyl radicals generated in the system (Chandrasekara & Shahidi, 2011a).

3.8. Correlation of analyses

A significant correlation ($r = 0.7250$, $p = 0.027$) existed between total phenolics and the scavenging activity against hydroxyl radicals. The higher significant correlation found between total phenolics ($r = 0.8782$, $p = 0.002$) and ORAC values as compared with the ones found against hydroxyl radicals suggests that phenolics from berry seed meals may act more effectively against peroxyl radicals rather than hydroxyl radicals in a concentration dependent manner. A higher significant correlation was found between total phenolics and reducing power ($r = 0.9822$, $p < 0.001$) as compared with the correlation found between chelating capacity and total phenolics ($r = 0.6860$, $p < 0.041$), thus indicating that in the Haber–Weiss cycle (Fenton reaction) in which ferric and ferrous ions are continuously being reduced and oxidised, phenolics from berry seed meals may act more effectively by reducing ferric ions rather than by chelating ferrous ions. A concentration dependent effect was observed between the inhibition of LDL-cholesterol oxidation and total phenolics as evidenced by their significant correspondent positive correlations ($r = 0.8312$, $p = 0.005$). Furthermore, the total phenolics also had a positive correlation with the inhibition of

DNA damage induced by both peroxyl ($r = 0.7340$, $p < 0.024$) and hydroxyl ($r = 0.8196$, $p < 0.007$) radicals. From the aforementioned, the total phenolic content may serve as a good predictor of activity against ROS, metal ions, and in biological model systems. However, an explanation at the molecular level would be desirable.

Protocatechuic acid had a positive correlation with ORAC ($r = 0.8767$, $p = 0.002$), reducing power ($r = 0.8774$, $p = 0.002$), chelating capacity ($r = 0.7430$, $p = 0.022$), and in biological model systems: copper induced LDL-cholesterol oxidation ($r = 0.8720$, $p = 0.002$), hydroxyl DNA induced oxidation ($r = 0.7632$, $p = 0.017$). Caffeic acid was positively correlated with ORAC ($r = 0.7796$, $p = 0.013$), reducing power ($r = 0.9540$, $p < 0.001$) and hydroxyl DNA induced oxidation ($r = 0.6796$, $p = 0.044$). Gallic hexoside correlated with ORAC ($r = 0.8658$, $p = 0.003$), reducing power ($r = 0.7363$, $p = 0.024$), and copper induced LDL-cholesterol oxidation ($r = 0.7049$, $p = 0.034$). Epicatechin correlated only with copper induced LDL-cholesterol oxidation ($r = 0.6924$, $p = 0.039$). Quercetin, however, positively correlated with ORAC ($r = 0.7051$, $p = 0.034$), reducing power ($r = 0.7548$, $p = 0.019$), copper induced LDL-cholesterol oxidation ($r = 0.6772$, $p = 0.045$), hydroxyl ($r = 0.6974$, $p = 0.037$) and peroxyl ($r = 0.6680$, $p = 0.049$) DNA induced oxidation. Epigallocatechin showed positive correlation with reducing power ($r = 0.8446$, $p = 0.0340$), copper induced LDL-cholesterol oxidation ($r = 0.8178$, $p = 0.047$), and hydroxyl DNA induced oxidation ($r = 0.8773$, $p = 0.022$). However, myricetin correlated only with ORAC ($r = 0.9274$, $p = 0.023$) and reducing power ($r = 0.9492$, $p = 0.014$). Quercetin pentose had positive correlations with ORAC ($r = 0.8845$, $p = 0.008$), reducing power ($r = 0.9523$, $p = 0.001$), copper induced LDL-cholesterol oxidation ($r = 0.8125$, $p = 0.026$), hydroxyl ($r = 0.8723$, $p = 0.010$) and peroxyl ($r = 0.7715$, $p = 0.042$) DNA induced oxidation. Procyanidin dimer B1 showed positive correlations with ORAC ($r = 0.8049$, $p = 0.009$), reducing power ($r = 0.8400$, $p = 0.005$), and copper induced LDL-cholesterol oxidation ($r = 0.6692$, $p = 0.049$) and procyanidin B3 showed positive correlation only with ORAC ($r = 0.7009$, $p = 0.035$) and reducing power ($r = 0.8593$, $p = 0.003$). Although four procyanidin isomers have been identified and quantified, only two of them (B1 and B3) showed a concentration dependent correlation with the antioxidant assays. Since all four have the same molecular weight this difference in their activity may be explained by stereochemical structures. In fact, our findings support the theory that not only the number of hydroxyl groups but also their positions may induce different effects in their bioactivities. Amongst anthocyanins only peonidin-3-*O*-glucoside showed significant positive correlations with reducing power ($r = 0.8082$, $p = 0.015$), chelating capacity ($r = 0.7818$, $p = 0.022$), copper induced LDL-cholesterol oxidation ($r = 0.8025$, $p = 0.017$), hydroxyl ($r = 0.8635$, $p = 0.006$) and peroxyl ($r = 0.7822$, $p = 0.022$) DNA induced oxidation.

4. Conclusion

The extraction yield of soluble phenolics (free and esterified) from berry seed meals was in the decreasing order of methanol–acetone–water > acetone–water > methanol–water > water. Further quantification of soluble free and esterified phenolics using HPLC-DAD-ESI-MS^n confirmed the highest efficiency of methanol–acetone–water as compared with acetone–water. Gallic acid and its derivative (gallic hexoside) were the major phenolic acids present. Quercetin, and its two derivatives (quercetin-3-*O*-glucuronide and quercetin pentose), were the major flavonoids in seed meals from blackberry and black raspberry, whereas procyanidin dimer B gave the highest contribution of total flavonoids in blueberry seed meals. The only anthocyanin identified in the blackberry and black raspberry seed meals was peonidin-3-*O*-glucoside; peonidin derivatives (peonidin-3-*O*-arabinoside and peonidin-3-*O*-glucoside) were the major anthocyanins blueberry seed meals. However, as mentioned before, the extraction system employed might have decomposed the existing anthocyanins and hence their contents might be underestimated. Blackberry seed meal was a richer source of phenolics compared to black raspberry and blueberry seed meals. The concentration of phenolic compounds in samples tested was in the order insoluble-bound > esterified > free form. Phenolics extracted neutralised peroxyl and hydroxyl radicals and had positive effects against metal ions. All fractions tested inhibited copper-induced LDL-cholesterol oxidation and DNA damage induced by both peroxyl and hydroxyl radicals. The highest inhibition was due to insoluble-bound phenolics from berry seed meals. The significant correlations found between the concentration of protocatechuic acid, gallic hexoside, epicatechin, quercetin, epigallocatechin, quercetin pentose, procyanidin dimer B1, and peonidin-3-*O*-glucoside in berry seed meals, and the ability of inhibiting the copper-induced LDL-cholesterol oxidation, indicates that these compounds may play an important role in preventing atherosclerosis and associated cardiovascular diseases. Furthermore, protocatechuic acid, caffeic acid, quercetin, epigallocatechin, quercetin pentose, and peonidin-3-*O*-glucoside correlated significantly with at least one of the mechanisms (hydroxyl or peroxyl induced oxidation) of DNA damage, indicating these compounds as being potentially responsible for inhibition of mutagenesis and further cancer initiation.

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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.2015.10.107.

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