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Water at room temperature as a solvent for the extraction of apple pomace phenolic compounds

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ABSTRACT

A fractionation method was used to extract phenolic compounds from apple pomace (AP) involving a first extraction with water and subsequent extractions of the same residue with two different organic solvents. The water extracts obtained contained high amounts of phenolic compounds with high antioxidant capacity. However, the second and third extractions of the same residue still extracted considerable amounts of remaining phenolic compounds, both with significant antioxidant capacities. Liquid chromatography–electrospray ionisation mass spectrometry (LC–ESI/MS) studies showed water to be a good solvent to extract hydroxycinnamic acids, flavonols, flavanols, dihydrochalcones and flavones present in the AP. However, water was not the ideal solvent to extract the quercetin glycosides.

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1. Introduction

Apple pomace (AP) is the main by-product of the cider industry and represents a serious environmental concern due to the vast amounts (millions of tonnes-EU) produced every year (Kennedy et al., 1999). AP is a heterogeneous mixture consisting of peel, core, seed, calyx, stem and soft tissue. Its composition varies according to the apple variety, agricultural practises, fruit maturity and the extraction process used to make cider (Kennedy et al., 1999) and particularly depends on the number of times the fruits are pressed (Vendruscolo, Albuquerque, Streit, Esposito, & Ninow, 2008).

Whole apples and their by-products such as AP are good sources of phenolic compounds (Schieber, Keller, & Carle, 2001; Tsao, Yang, Young, & Zhu, 2003). Phenolic compounds are known as free radical scavengers and therefore can act as potential disease-preventing agents against a range of degenerative diseases such as cardiovascular disease, cancer, inflammation, arthritis, immune system decline, brain dysfunction and cataracts (Lu & Foo, 1997, 2000). The phenolic compounds present in AP are mainly phenolic acids and flavonoids. To date, the major class of compounds identified in AP are flavonoids, where flavonols are the largest sub-class followed by flavanols, flavanones, flavones, dihydrochalcones and anthocyanins. The phenolic acids identified are primarily hydroxycinnamic acid derivatives and lesser of hydroxybenzoic acids (Cam & Aaby, 2010; Cetkovic et al., 2008; Diñeiro García, Valles, & Picinelli Lobo, 2009; Foo & Lu, 1999; Lu & Foo, 1997, 2000; Sanchez-Rabaneda et al., 2004; Schieber et al., 2003; Suárez et al., 2010).

Typical extraction procedures of phenolic compounds from AP are mostly carried out using organic solvents, such as 70% acetone or 80–100% methanol (Cetkovic et al., 2008; Diñeiro García et al., 2009; Foo & Lu, 1999; Lu & Foo, 1997, 2000; Schieber et al., 2003; Suárez et al., 2010). Sanchez-Rabaneda et al. (2004) have used fractionation extraction with ethyl acetate and dichloromethane. Some recent studies have reported extraction of the phenolic compounds using alternatives to organic solvents, such as sub-critical extraction (Adil, Cetin, Yener, & Bayindirli, 2007), pressurised liquid extraction (Wijngaard & Brunton, 2009) and water (Cam & Aaby, 2010). The latest review (Wijngaard, Hossain, Rai, & Brunton, 2012) provides an excellent overview of various solvents/techniques used to date in the extraction of phenolic compounds from by-products of food plants.

Analytical techniques used for separation and identification are an important aspect of a successful study of phenolic compounds. The liquid chromatography–diode array detector (LC–DAD) is the most common method, but it has the drawback of requiring known standards (not available for all phenolics) to validate identification (Cetkovic et al., 2008; Diñeiro García et al., 2009; Schieber et al., 2003; Suárez et al., 2010). Use of a mass spectrometer, which is a universal detector, coupled to the LC–DAD can bypass the need for standards, especially for simple phenolic compounds (Cam & Aaby, 2010). As many as 60 phenolic compounds have been identified in AP using LC–MS/MS (Sanchez-Rabaneda et al., 2004). MS/MS, or tandem mass spectrometry, is a powerful analytical

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technique that can provide primary structural information of molecules and can further aid in establishing the chemical identity of molecules. Identification of phenolic compounds is achieved based on three different parameters, namely the *m/z* ratio, the retention time and the fragmentation pattern; thus identification is no longer limited by the availability of commercial standards. NMR spectroscopy has also been used in the identification of phenolic compounds in AP, but it inherently requires purified compounds of adequate concentration (Foo & Lu, 1999; Lu & Foo, 1997).

Some phenolic compounds identified in AP have been correlated with antioxidant capacities using various methods (DPPH, hydroxyl and superoxide anion radical scavenging activity, FRAP) thus establishing the fact that AP is a valuable source of antioxidants. High correlations between the antiradical activities, total phenolics, total flavonoids, total flavanols and some individual phenolic compounds have been reported (Cetkovic et al., 2008). A predicted model has also been developed to predict antioxidant activity as a function of the phenolic profile. The antioxidant activity measured by DPPH and FRAP assays could be predicted by the contents of phloridzin, procyanidin B2, rutin, isoquercetrin, protocatechuic acid and hyperin (Diñeiro García et al., 2009). It has been shown that epicatechin, polymers of procyanidin, quercetin glycosides, chlorogenic acid, phloridzin and 3-hydroxyphloridzin showed DPPH radical scavenging activities 2–3 times and superoxide anion radical scavenging activities 10–30 times higher than those of vitamins C and E (Lu & Foo, 2000). This study also reported that the presence of lower molecular weight procyanidins and the quercetin glycosides showed excellent activity in the DPPH and superoxide anion radical scavenging activity assays.

Much work has been done on phenolic compounds of AP and efforts are being made to improve the extraction of phenolic compounds by using healthy and environmentally friendly methods (Adil et al., 2007; Cam & Aaby, 2010; Wijngaard & Brunton, 2009, 2010). The use of water is a good choice for the extraction of phenolic compounds due to its safety, accessibility and low cost. Some research has been reported evaluating the extraction of phenolic compounds with water at 100 °C (Cam & Aaby, 2010), although it must be noted that using high temperatures adds significant cost for industry. Therefore, the aim of this work was to evaluate the efficiency of water at room temperature for the extraction of AP phenolic compounds.

2. Materials and methods

2.1. Chemicals

Acetic acid, aluminium chloride, ascorbic acid, β-carotene, catechin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride, Folin Ciocalteu's phenol reagent, gallic acid, linoleic acid, sodium acetate, sodium carbonate, sodium nitrite, quercetin, 2,4,6-Tris(2-pyridil)-s-triazine (TPTZ), Tween 20 and vanillin were purchased from Sigma–Aldrich. Hydrochloric acid was purchased from AlfaAesar and sodium hydroxide from Applichem. All organic solvents were of analytical grade and obtained from Fisher Chemical. The solvents used for sample preparation of LC–MS studies were HPLC grade, and the water (18.2 MΩ) was purified on a Millipore Direct-Q system (Millipore Ireland, Cork, Ireland). Fifteen standard phenolic compounds (>97% purity), namely chlorogenic acid, epicatechin, isorhamnetin, rhamnetin, kaempferol luteolin, procyanidin trimer C, phloretin, phloridzin, quercetin, quercetin-3-O-arabinoside, quercetin-3-O-galactoside, quercetin-3-O-glucoside, quercetin-3-O-rhamnoside and rutin were purchased from Sigma Ireland (Arklow, Co. Wicklow, Ireland).

2.2. Apple pomace

Apple pomace (AP) was provided by Bulmers Limited (Clonmel, Ireland). On arrival, the samples were packed under vacuum to prevent oxidation and fermentation and stored at −20 °C until being freeze dried. The freeze dried AP was coarsely ground and passed through 250 μm sieve and stored in polyethylene bags at −20 °C for further analysis.

2.3. Extraction of phenolic compounds

The extraction of phenolic compounds was performed by a fractionation method based on a method previously described (Ferreira et al., 2002). This method was modified according to the nature of the sample and the aim of the study. The freeze dried and ground samples (3 g) were stirred with 40 mL water three times (40 min, 40 min, 10 min) at room temperature. In each extraction, the water extracts were filtered and the three collected filtrates were combined. The residual pomace was then reconstituted in methanol (20–100%) and stirred as above. The procedure was repeated with acetone (20–100%) on the residue left after the methanol extract. All extractions were carried out in acidic conditions (addition of glacial acetic acid at 5 mL/L) to prevent oxidation of the phenolic compounds. The resultant three crude extracts were classified as water extract (WE), methanol extract (ME) and acetone extract (AE). Solid phase extraction (SPE) with C_{18} cartridges (DSC-18, Supelco) was performed to select the organic compounds from the crude extract and to remove the sugars from each extract which would otherwise have interfered with the Folin assay. The sugars were eluted from the cartridge with 300 mL of 2% acetic acid and the phenolic-rich fractions were eluted with methanol containing 0.1% HCl. The phenolic fractions from WE, ME and AE were concentrated using a rotary evaporator at 40 °C, frozen at −70 °C and freeze dried.

2.4. Determination of phenolic compounds

2.4.1. Total phenolic content (TPC)

Total phenolics were determined using the Folin–Ciocalteu assay (Ganesan, Kumar, & Bhaskar, 2008). The reaction mixture was prepared by mixing 100 μL extract with 2 mL of 2% Na_2CO_3. The mixture was allowed to stand for 2 min at room temperature followed by the addition of 100 μL of Folin Ciocalteu's phenol reagent (1:2). After 30 min incubation at room temperature in the dark the absorbance was measured at 720 nm and the results were expressed in gallic acid equivalents.

2.4.2. Total flavonoid content (TFC)

Total flavonoids were estimated according to the method previously described (Liu, Lin, Wang, Chen, & Yang, 2009). An aliquot of 250 μL extract was mixed with 1.25 mL of distilled water and 75 μL of 5% $NaNO_2$. After 6 min, 150 μL of 10% $AlCl_3$ was added. Finally, 500 μL of 1 M NaOH was added and the total volume was made up to 2.5 mL with distilled water. Absorbance was measured at 510 nm. Results were expressed in quercetin equivalents.

2.4.3. Proanthocyanidins content (PAC)

Proanthocyanidins were determined using the vanillin assay previously described (Sun, Ricardo-da-Silva, & Spranger, 1998). An aliquot of 0.5 mL extract was mixed with 3 mL of 4% vanillin–methanol solution and 1.5 mL HCl. Absorbance was measured after 15 min at 500 nm. Results were expressed in catechin equivalents.

2.5. Antioxidant capacity evaluation

2.5.1. DPPH radical scavenging activity

The DPPH assay was performed in a 96-well round-bottom microplate (Sarstedt, Inc, USA) according to the method previously described by other authors (Yen & Chen, 1995). Three wells of the microplate were loaded with 50 μL of extract and 200 μL of DPPH (test), another three wells were filled with 50 μL of solvent and 200 μL of DPPH (control) and one well with 50 μL of extract and 200 μL of extractant solvent (blank). DPPH solution (0.5 mg/mL) was freshly prepared each day and a dilution of 1:10 from this solution was used in the reaction mixture. The microplate was incubated in a microplate reader (Synergy HT, Biotek, VT, USA) for 30 min at 25 °C and the absorbance was measured at 517 nm every 5 min. The ability to scavenge the DPPH radical was calculated using the following equation: $((Abs_{control} - Abs_{test}) \times 100)/Abs_{control}$. Different concentrations of some extracts were tested to determine the EC_{50} value. EC_{50} value is defined as the concentration of extract required for 50% scavenging of DPPH radical under the experimental conditions employed (Cetkovic et al., 2008).

2.5.2. Ferric reducing antioxidant power (FRAP)

FRAP assay was performed according to the method described by Diñeiro García et al. (2009). An aliquot of 30 μL of extract was mixed with 90 μL of distilled water and 900 μL of the FRAP reagent. This reaction mixture was incubated at 37 °C for 2 h. Absorbance was measured at 595 nm and the results expressed as ascorbic acid equivalents. The FRAP reagent was prepared freshly each day by mixing 2.5 mL of TPTZ (10 mM in 40 mM hydrochloric acid), 2.5 mL of ferric chloride (20 mM) and 25 mL of sodium acetate buffer (300 mM, pH 3.6).

2.5.3. β-Carotene/linoleic acid system

β-Carotene/linoleic acid assay was carried out according to the method described by Lu and Foo (2000). 1 mL of β-Carotene in chloroform (3.34 mg/mL) was added into a round-bottom flask containing 40 mg linoleic acid and 400 mg Tween 20. The chloroform was removed by rotary evaporation and 100 mL of oxygenated distilled water was added slowly with vigorous agitation and placed in an ultrasound bath to form an emulsion. An aliquot of 1.44 mL emulsified solution was added into a microtube containing 60 μL extract. The absorbance was measured immediately at 470 nm against a blank consisting of the emulsion without β-carotene. The microtubes were placed in a water bath at 40 °C and the absorbance was measured every 15 min.

2.6. Liquid chromatography–electrospray ionisation mass spectrometry (LC–ESI/MS)

LC–ESI/MS was performed on a Q-Tof Premier mass spectrometer (Waters Corp., Micromass MS Technologies, Manchester, UK), coupled to an Alliance 2695 HPLC system (Waters Corp., Milford, MA). The Q-Tof Premier was equipped with a lockspray source where an internal reference compound (leucine–enkephalin) was introduced simultaneously with the analyte for accurate mass measurements. Compounds were separated on an Atlantis T3 C_{18} column (Waters Corp., Milford, MA; 100 mm × 2.1 mm; 3 μm particle size) using 0.5% aqueous formic acid (solvent A) and 0.5% formic acid in 50:50 (v/v) acetonitrile:methanol (solvent B). Column temperature was maintained at 38 °C. A stepwise gradient from 10 to 95% solvent B was applied at a flow rate of 0.2 mL/min for 30 min. Electrospray mass spectra data were recorded on a negative ionisation mode for a mass range from *m/z* 100 to 1600. Capillary voltage and cone voltage were set at 3 kV and 30 V, respectively. Collision-induced dissociation (CID) of the analytes was achieved using 10–30 eV of energy using argon as the collision gas.

Primary stock solutions of the 15 standard phenolic compounds were prepared in methanol at concentrations of 1 mg/mL and stored at −20 °C. Four groups of intermediate working standard mix solutions: (Group 1) 500 μg/mL each of chlorogenic acid, epicatechin, quercetin, quercetin-3-O-galactoside and quercetin-3-O-rhamnoside; (Group 2) 200 μg/mL each of kaempferol, quercetin-3-O-arabinoside, quercetin-3-O-glucoside and rutin; (Group 3) 100 μg/mL each of kaempferol, luteolin, phloretin and phloridzin and (Group 4) 50 μg/mL each of isorhamnetin and rhamnetin were prepared in methanol and stored at −20 °C. Accordingly seven calibrants of 2, 5, 8, 10, 12, 15, 20 μg/mL (Group 1); 0.8, 2, 3.2, 4, 4.8, 6, 8 μg/mL (Group 2); 0.4, 1, 1.6, 2.0, 2.4, 3, 4 μg/mL (Group 3); 0.2, 0.5, 0.8, 1.0, 1.2, 1.5, 2.0 μg/mL (Group 4) and 2, 4, 10, 16, 20, 24 and 30 μg/mL of procyanidin C were prepared. A low and a medium concentrated standard samples each was prepared as controls. Quanlynx software supplied with the mass spectrometry software Masslynx 4.1 (Waters Corporation, Milford, USA) was used to aid the quantification of the phenolic compounds in the analytes.

2.7. Response surface methodology (RSM)

A central composite rotatable design was used to investigate the effects of two independent variables, concentration of solvent (X_1) and extraction time (X_2), on AP total phenolic content (TPC), total flavonoid content (TFC) and the antioxidant capacity measured by DPPH and FRAP. Results from preliminary trials were used to select suitable values for the independent variables. A second order polynomial Eq. (1) for the dependent variables was established to fit the experimental data. An analysis of variance (ANOVA) was carried out using STATGRAPHICS (Centurion XV.II 2006) to determine the significance levels of variables.

$$Y = \beta 0 + \beta 1X_1 + \beta 2X_2 + \beta 11X_1^2 + \beta 22X_2^2 + \beta 12X_1X_2 \quad (1)$$

where $X_1, X_2 \ldots X_1X_2$ are the independent variables with their linear, quadratic and interactive models, $\beta 0, \beta 1, \beta 2 \ldots \beta 12$ are the regression coefficients of responses.

2.8. Statistical analysis

All TPC, TFC, DPPH and FRAP measurements were carried out in three independent extractions and performed in triplicate for each extraction. Data were reported as mean ± standard deviation (SD). To test the significance of differences between means, analysis of variance (ANOVA) was used. Differences were considered to be statistically significant at $p \leqslant 0.05$.

3. Results and discussion

3.1. Preliminary results

The phenolic compounds of AP were extracted by a fractionation method involving a first extraction with water and a second and third extraction of the same residue with organic solvents, namely methanol and acetone. The aim was to see if after the extraction with water there were any phenolic compounds remaining in the residue responsible for significant antioxidant effects. To achieve this, preliminary work was done to optimise the water extraction and the subsequent extractions of the residue with methanol and acetone. Water extraction was optimised for amount of sample, solvent and extraction time in the range 3–7 g of AP, 40–60 mL of water and 50–90 min of extraction. The data (not shown) revealed by ANOVA that the values obtained for yield using different amounts of sample and solvent were not

Table 1
Experimental design and corresponding response values for methanol (ME) and acetone (AE) extracts.

ME	X_1	X_2	Y_1	Y_2	Y_3	Y_4	Y_5
1	88 (2)	70 (0)	0.95	48	250	21	14
2	60 (0)	98 (2)	0.12	267	659	95	468
3	60 (0)	70 (0)	0.08	231	630	93	411
4	40 (−1)	90 (1)	0.06	393	1036	100	557
5	60 (0)	42 (−2)	0.12	152	391	88	263
6	40 (−1)	50 (−1)	0.06	277	588	85	299
7	32 (−2)	70 (0)	0.13	1711	852	90	549
8	60 (0)	70 (0)	0.07	226	645	94	328
9	80 (1)	50 (−1)	0.25	46	355	41	32
10	80 (1)	90 (1)	0.23	50	301	53	54
AE							
1	60 (0)	42 (−2)	0.15	73	416	38	22
2	88 (2)	70 (0)	0.04	51	287	25	9
3	60 (0)	70 (0)	0.15	59	331	36	17
4	60 (0)	70 (0)	0.12	66	325	41	24
5	40 (−1)	50 (−1)	0.11	311	735	87	379
6	32 (−2)	70 (0)	0.17	319	960	99	368
7	60 (0)	98 (2)	0.11	79	369	81	27
8	80 (1)	90 (1)	0.49	43	165	11	0
9	40 (−1)	90 (1)	0.12	319	810	100	376
10	80 (1)	50 (−1)	0.42	51	277	16	6

$Y1$ = Yield (%); $Y2$ = TPC (μg gallic acid/mg dry extract); $Y3$=TFC (μg quercetin/mg dry extract); $Y4$ = DPPH (% inhibition at 1 mg/mL after 30 min); $Y5$ = FRAP (μg ascorbic acid/mg dry extract).

significantly different ($p \leqslant 0.05$). For that reason, the lower amounts of sample and solvent (3 g of AP and 40 mL of water, repectively) were chosen. The best yields were obtained with an extraction time of 90 min.

The subsequent extractions with methanol and acetone were also optimised for solvent concentration and extraction time. The optimisation was carried out using RSM and the experimental design for both extractions and corresponding response values are presented in Table 1. A regression analysis was carried out to fit mathematical models to the experimental data and the regression coefficients for the uncoded variables are shown in Table 2. The regression models fit significantly (R^2) for all independent variables except yield of acetone. The result obtained for yield of acetone is probably due to this being the final extraction of the residue and so the residual quantities of compounds are too small for accurate analysis. The p-values of regression and ANOVA analysis showed that solvent concentration is the main variable for all the responses. Extraction time also affects significantly TFC in both methanol and acetone extracts and DPPH and FRAP in methanol extracts. The quadratic factor of solvent concentration affects TPC, TFC and FRAP in acetone extracts and DPPH in methanol extracts. The interactions affected TFC in both methanol and acetone extracts.

The estimated response surfaces based on the experimental data are represented in Fig. 1 for each response for both methanol and acetone extraction. It can be seen that yield of extraction increases with the increase of solvent concentration. Inverse results were obtained for the content of phenolics (TPC, TFC) and for the antioxidant capacities (DPPH, FRAP). Therefore, if concentrations of methanol and acetone are increased, this may lead to the extraction of unknown compounds without antioxidant capacity. Combining the optimum value for each response, making use of the predicted equations and aiming for high content of phenolic compounds and antioxidant capacity we choose 90 min for both extractions and 40% methanol and 40% acetone to extract the residue left after water extraction.

3.2. Quantification of phenolic compounds

Fractionation of 1 kg of dried AP yielded extracts equivalent to 2.566 g of gallic acid, 6.696 g of quercetin and 837 mg of catechin (Fig. 2). Water first extracted 67% of the total phenolic compounds, 72% of the total flavonoids and 51% of PAC. This was followed by additional extraction of 17% and 16% TPC, 10% and 18% of TFC and 21% and 28% of the PAC when using 40% methanol and 40% acetone, respectively.

The results obtained for TFC in ME were lower than those obtained for AE, though TPC was similar ($p \leqslant 0.05$) to AE, thus suggesting the extraction of other types of phenolic compounds. The higher TFC extracted with AE when compared to ME may be explained by the higher efficiency of flavonoid extraction as reported by Suárez et al. (2010) which could be flavanols as indicated by the higher values obtained for PAC.

In this fractionation method, water extracted the highest amount of TPC, TFC and PAC. However, subsequent extractions using aqueous solutions (40%) of methanol and acetone continued to extract the remaining TPC, TFC and PAC in considerable amounts.

3.3. Antioxidant capacity evaluation

The % inhibition of DPPH after 30 min reaction with different concentrations of WE, ME and AE is presented in Fig. 3. The results showed that DPPH radical scavenging activity efficiencies were in the order of WE ⩾ ME > AE. The EC_{50} values were 82.0 ± 8.0 μg/mL for WE; 94.1 ± 10.0 μg/mL for ME and 115.4 ± 18.0 μg/mL for AE. These results are quite low when compared to previously reported figures (6.33–15.72 mg/mL) for 80% methanol extracts of AP (Cetkovic et al., 2008). The results suggest that extracts obtained in this study exhibit a high antioxidant capacity at lower concentrations.

Table 2
Regression coefficients and analysis of variance of uncoded units for dependent variables methanol (ME) and acetone (AE) extracts.

		β0	β1	β2	β11	β12	β22	R^2
ME	Y_1	0.873	−0.046	0.008	0.000	0.000	0.000	77.8
	Y_2	1846	−84.6	40.2	0.593	−0.070	−0.244	70.8
	Y_3	−1244	21.2	40.5	−0.088	−0.314	−0.121	99.3
	Y_4	−63.0	4.93	1.02	−0.050	−0.002	−0.005	98.9
	Y_5	−815	20.6	20.8	−0.165	−0.147	−0.060	95.1
AE	Y_1							
	Y_2	1262	−27.2	−6.13	0.185	−0.010	0.048	94.1
	Y_3	2318	−48.1	−3.94	0.362	−0.117	0.073	99.5
	Y_4	256	−3.85	−1.93	0.025	−0.011	0.022	92.2
	Y_5	1710	−39.2	−7.93	0.262	−0.002	0.057	92.1

$Y1$ = Yield (%); $Y2$ = TPC (μg gallic acid/mg dry extract); $Y3$ = TFC (μg quercetin/mg dry extract); $Y4$ = DPPH (% inhibition at 1 mg/mL after 30 min); $Y5$ = FRAP (μg ascorbic acid/mg dry extract).

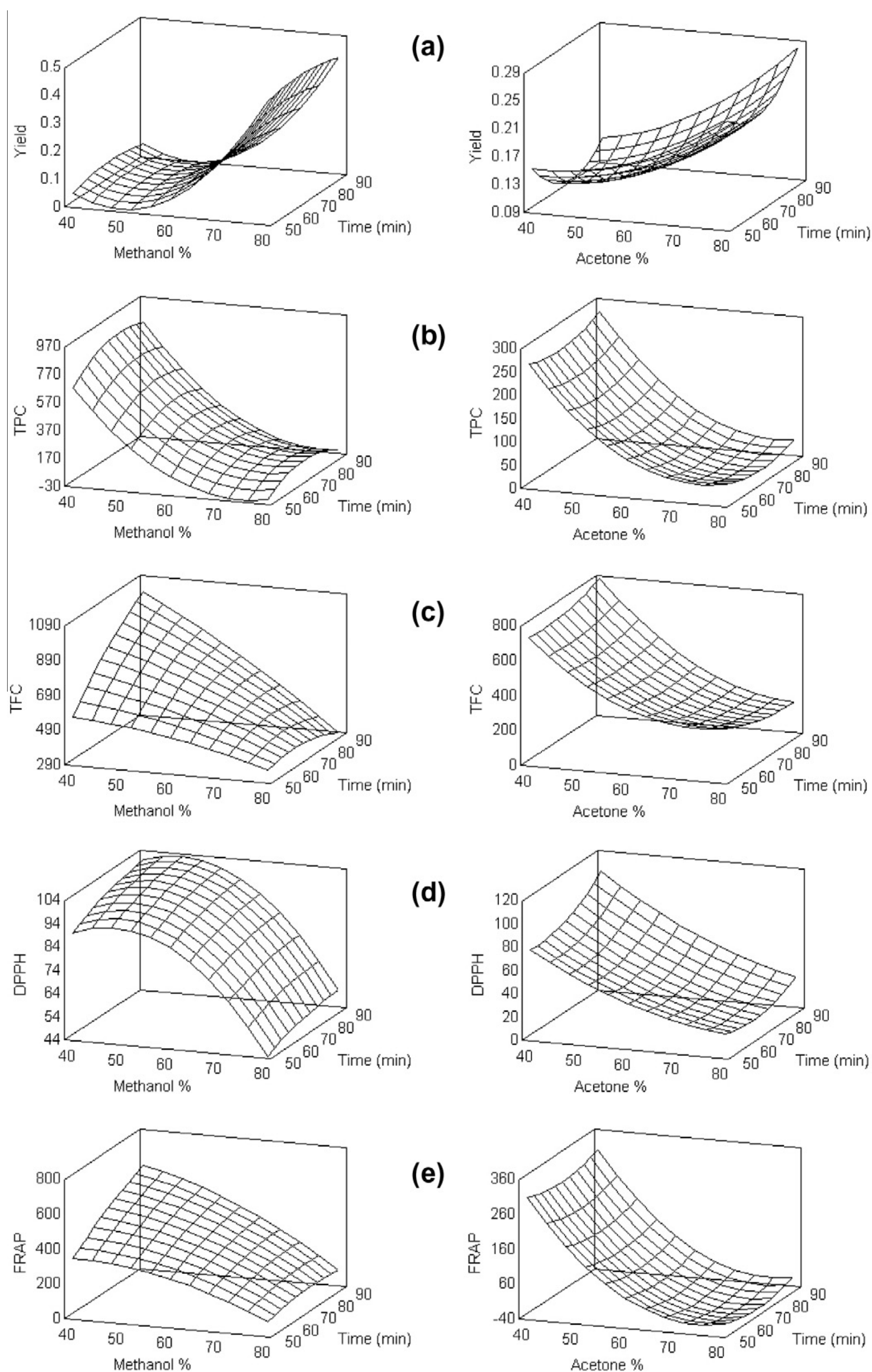

Fig. 1. Estimated response surfaces for the effect of solvent concentration and extraction time on (a) yield, (b) TPC, (c) TFC, (d) DPPH and (e) FRAP in methanol and acetone extracts.

The results obtained for FRAP in WE, ME and AE showed that 1 kg of dried AP had FRAP equivalent to 1.718 g of ascorbic acid which is the equivalent to 1.169 g, 0.288 g and 0.261 g of ascorbic acid upon using water, methanol and acetone extraction, respectively (Fig. 4). ME and AE showed lower FRAP values than did the WE and there were no significant differences ($p \leqslant 0.05$) between the FRAP values of ME and AE. The total FRAP value was lower than has been reported in other studies, where 70% acetone extracts

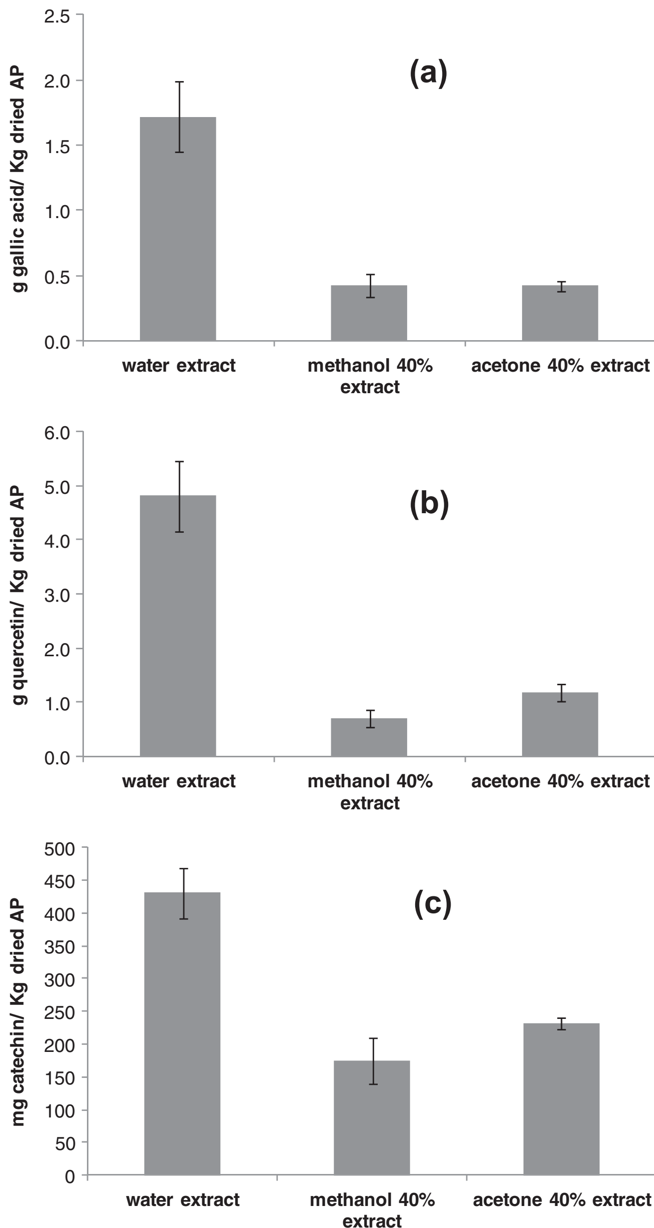

Fig. 2. Results of (a) TPC, (b) TFC and (c) PAC in water, methanol and acetone extracts.

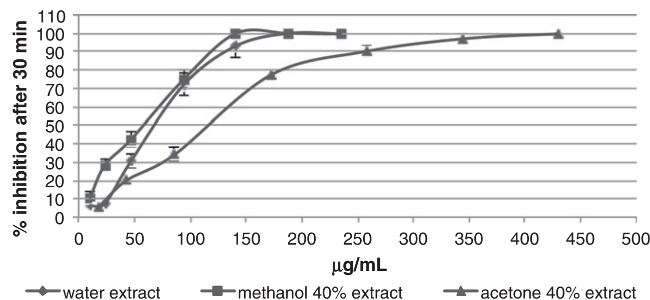

Fig. 3. DPPH radical scavenging activity for different concentrations of water, methanol and acetone extracts using optimum conditions of RSM.

resulted in 4.1–14.5 g ascorbic acid/kg dried AP (Diñeiro García et al., 2009; Suárez et al., 2010) and 80% methanol extracts resulted in 7.73 g ascorbic acid/kg dried AP (Suárez et al., 2010). The variations in the results could be due to the different methods of phenolic extraction.

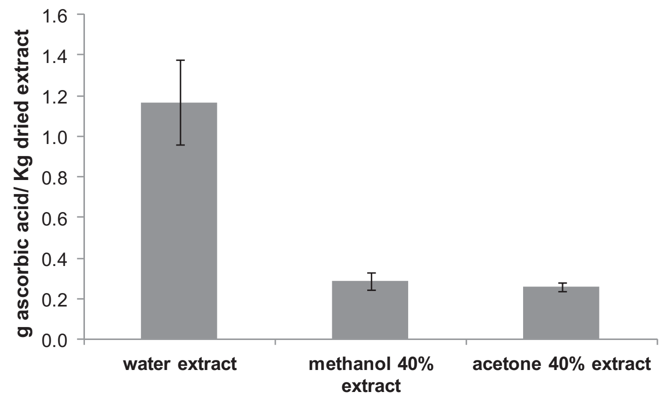

Fig. 4. FRAP in water, methanol and acetone extracts using optimum conditions of RSM.

The % of preservation of β-carotene in a β-carotene/linoleic acid system during 90 min reaction with WE, ME and AE is shown in Fig. 5. WE after 90 min reaction showed the highest β-carotene preservation capacity (60%) when compared to ME and AE (25–30%).

The highest antioxidant capacity of WE measured by FRAP and by the β-carotene/linoleic acid system can be explained by the higher amounts of phenolic compounds found in WE. The similar results observed for DPPH in WE and ME suggest that the type of compounds extracted in the second step of the fractionation method are very efficient against DPPH radical scavenging activity.

3.4. Identification of phenolic compounds by LC–ESI/MS

Phenolic acids and flavonoids were the two main families of phenolic compounds identified in the AP extracts, as previously reported (Cam & Aaby, 2010; Cao, Wang, Pei, & Sun, 2009; Cetkovic et al., 2008; Diñeiro García et al., 2009; Sanchez-Rabaneda et al., 2004; Schieber et al., 2003; Suárez et al., 2010). Accurate mass measurements and tandem mass spectrometry were applied to identify each of the phenolic compounds (Table 3). Phenolic acids, in particular hydroxycinnamate derivatives (i.e. chlorogenic acid or caffeoylquinic acid and feruloylquinic acid), were found largely in WE. As far as we are aware this is the first report on the presence of feruloylquinic acid in AP. Flavonoids in AP were composed of seven flavonols, five flavanols, three dihydrochalcones and one flavone. The seven flavonols were found in all the three extracts. The flavanols were, however, distributed unequally in the three

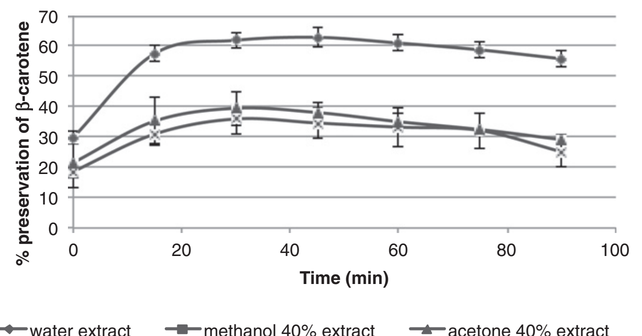

Fig. 5. β-carotene/linoleic acid system in water, methanol and acetone extracts using optimum conditions of RSM.

Table 3
List of identified compounds in AP.

Compound	MW	Obs m/z	Calcd m/z	Major fragments m/z	Molecular formula
Phenolic acids					
Hydroxycinnamic acids					
Chlorogenic acid[a,b]	354	353.0872	353.0873	191.1, 179.0, 173.0	$C_{16}H_{18}O_9$
Feruloylquinic acid	368	367.1030	367.1029	179.0, 135.0	$C_{17}H_{20}O_9$
Flavonoids					
Flavonols					
Quercetin[a,b]	302	301.0341	301.0348	227.1, 151.1	$C_{15}H_{10}O_7$
Isorhamnetin[a,b]	316	315.0497	315.0505	300.0, 151.0	$C_{16}H_{12}O_7$
Quercetin 3-O-arabinoside[a,b]	434	433.0762	433.0771	300.1	$C_{20}H_{18}O_{11}$
Quercetin 3-O-glucoside[a,b]	464	463.0878	463.0877	300.0, 151.0	$C_{20}H_{18}O_{11}$
Quercetin-3-O-rhamnoside[b]	448	447.0925	447.0927	300.1, 151.0	$C_{21}H_{20}O_{11}$
Quercetin 3-O-galactoside[a,b]	464	463.0878	463.0877	300.0, 151.0	$C_{21}H_{20}O_{12}$
Quercetin-3-O-rutinoside (rutin) [ab]	610	609.1456	609.1456	463.1, 300.1	$C_{27}H_{30}O_{16}$
Flavanols					
Epicatechin[a,b]	290	289.0715	289.0712		$C_{15}H_{14}O_6$
Procyanidin dimer A2[b]	576	575.1167	575.1190		$C_{30}H_{23}O_{12}$
Procyanidin dimer B1 or B2[b]	578	577.1334	577.1346	289.1, 407.1, 125.0	$C_{30}H_{26}O_{12}$
Procyanidin trimer C[a,b]	866	865.1970	865.1980		$C_{45}H_{38}O_{18}$
Procyanidin tetramer D[b]	1154	1153.2655	1153.2614		$C_{60}H_{50}O_{24}$
Dihydrochalcones					
Phloretin[a,b]	274	273.0758	273.0763	273.1, 167.0	$C_{15}H_{14}O_5$
Phloridzin[a,b]	436	435.1287	435.1291	273.0, 167.0	$C_{21}H_{24}O_{10}$
Phloretin 2′-O-xylosil-glucoside[b]	568	567.1702	567.1714	273.1, 167.0	$C_{26}H_{32}O_{14}$
Flavones					
Kaempferol[a,b]	286	285.0392	285.0399	285.0	$C_{15}H_{10}O_6$

[a] Confirmed with commercial standards.
[b] Already identified in AP.

solvent extracts. The three dihydrochalcones and flavone (kaempferol) were present in all the three solvent extracts.

In order to obtain an understanding of the effect of various solvent constituents on the extraction of the major types of phenolic compounds, a majority of the phenolic compounds identified were quantified using a set of standard calibrants, as described earlier (Section 2.6). Due to identical elution time, procyanidin trimer C was used as a calibrant for the quantification of procyanidin dimer B. For the quantification of feruloylquinic acid, a similar structure and a compound from the same class of the phenolic acids, i.e., chlorogenic acid, was used as the calibrant. The results of the quantification study are presented on Table 4. The low and medium control standards in general showed acceptable range of concentration except for those compounds with elution times close to one another and with broad peaks such as procyanidins/epicatechin and rutin/quercetin-3-O-glucoside. The findings from this quantification study showed that water is the best extractant for phenolic acids and epicatechin, while 40% methanol is best for extracting flavones, flavonols and dihydrochalcones. The data further support previous reports where chlorogenic acid was

Table 4
Quantities of phenolic compounds in various extracts.

RT (min)	Compound	R^2	Controls		M40 (μg/mL)	Water (μg/mL)	A40 (μg/mL)
			Low μg/mL (expected)	Medium μg/mL (expected)			
	Phenolic acids						
	Hydroxycinnamic acids						
1.95	Chlorogenic acid	0.9908	3.5 (4.0)	5.5 (6.0)	5.32	7.28	–
4.04	Ferluoylquinic acid	0.9908	–	–	1.69	2.83	–
	Flavonoids						
	Flavonols						
12.95	Isorhamnetin	0.9924	0.47 (0.4)	0.95 (0.8)	0.46	0.11	0.47
11.01	Quercetin	0.9835	0.87 (0.8)	1.9 (1.5)	3.96	0.99	0.13
8.59	Quercetin-3-O-rhamnoside	0.9854	3.88 (4.0)	8.14 (8.0)	2.88	–	–
7.52	Quercetin 3-O-arabinoside	0.9733	1.57 (1.6)	3.37 (3.0)	0.71	–	–
6.13	Quercetin 3-O-glucoside	0.9896	1.4 (1.6)	3.4 (3.0)	3.71	0.23	0.78
5.4	Quercetin-3-O-rutinoside (rutin)	0.9722	1.59 (1.6)	3.71 (3.0)	8.92	1.57	1.51
	Flavanols						
2.28	Epicatechin	0.9798	4.2 (4.0)	10.0 (8.0)	3.7	5.39	4.3
2.02	Procyanidin trimer C	0.9981	4.8 (5.0)	11.2 (12.0)	–	2.84	3.15
2.02	Procyanidin dimer B	0.9981	–	–	–	6.99	5.6
	Dihydrochalcones						
12.14	Phloretin	0.9863	0.9 (0.8)	1.9 (1.5)	3.43	0.1	1.87
9.36	Phloridzin	0.9777	0.86 (0.8)	2 (1.5)	2.38	0.61	0.16
8.18	Phloretin 2′-O-xylosil-glucoside	–	–	–	–	+++	+
	Flavones						
12.51	Kaempferol	0.9863	0.9 (0.8)	1.8 (1.5)	1.98	0.21	0.36

observed as the main phenolic acid (Diñeiro García et al., 2009; Schieber et al., 2003; Suárez et al., 2010). The quantification results also showed that water readily extracted quercetin, but was a poor extractant of quercetin-mono-glycosides. Instead, 40% aqueous methanol (M_{40}) proved to be the best solvent to extract quercetin-mono-glycosides. This suggests that the quercetin-mono-glycosides are probably linked by hydrophobic interactions to cell walls and therefore are more difficult to extract from the AP with water. Similarly, of the flavonols, epicatechin was found in high amount in the water extract, while the polymers of procyanidins were present in water and 40% acetone extracts in almost equal amounts. This confirms the ability of acetone to extract procyanidins efficiently (Suárez et al., 2010). Only phloretin 2′-O-xylosyl-glucoside could not be quantified due to its low levels, however, its presence in water extracts was 3× the amount present in the 40% acetone extracts. Phloretin constituted the major form of the two dihydrochalcones quantified in AP which is in agreement with findings from other authors (e.g., Diñeiro García et al., 2009; Schieber et al., 2003; Suárez et al., 2010).

The other interesting observation from the quantification studies is that the phenolic acids and flavanols in Table 4 showed a similar trend to the TPC and PAC respectively (data shown in Fig. 2). The only major difference was that the TFC in water extract (Fig. 2b) was highest, in contrast to the low levels of flavonol-mono-glycosides of the same water extract compared to those in the methanol and acetone extracts. One viable argument could be that the TFC represents generally the aglycone flavonoids.

Water showed to be the best solvent to extract hydroxycinnamic acids and flavanols and showed reasonable extracting abilities for dihydrochalcones and flavones present in AP. However, subsequent extractions with aqueous solutions of methanol and acetone maximise the extraction of the same compounds, particularly flavonols and flavanols.

4. Conclusion

Phenolic compounds of AP, mainly phenolic acids and flavonoids, that display antioxidant activity are readily extracted with water and with food-compatible aqueous organic solutions. Water was shown to be a good solvent to extract the considerable amount of phenolic compounds present in AP and the major compounds extracted were chlorogenic acid, feruloylquinic acid, epicatechin, and procyanidins. Feruloylquinic acid has been identified for the first time in AP. Water is environmentally friendly and cheap, making it an ideal solvent for the extraction of AP phenolic compounds. However, in order to maximise the recovery of phenolic compounds from AP, the subsequent use of aqueous organic solvents such as methanol and acetone is suggested. The use of 40% aqueous methanol and 40% aqueous acetone proved to be more efficient extractants for quercetin mono-glycosides and flavanols respectively. Nevertheless, the content of organic and less environmentally friendly solvents can be minimized to 40%, unlike previously reported use of as high as 70% acetone and 80% methanol.

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