View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Food Chemistry 149 (2014) 151–158

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Optimisation of the extraction of phenolic compounds from apples using response surface methodology

Aline Alberti [a], Acácio Antonio Ferreira Zielinski [a], Danianni Marinho Zardo [c], Ivo Mottin Demiate [b], Alessandro Nogueira [b,*], Luciana Igarashi Mafra [a]

[a] Food Engineering Graduate Programme, Federal University of Paraná, Francisco H. dos Santos Street, CEP 81.531-990 Curitiba, Paraná, Brazil
[b] Food Science and Technology Graduate Programme, State University of Ponta Grossa, 4748 Carlos Cavalcanti Av., Uvaranas Campus, CEP 84.030-900 Ponta Grossa, Paraná, Brazil
[c] Pharmaceutical Sciences Department, State University of Ponta Grossa, 4748 Carlos Cavalcanti Av., Uvaranas Campus, 84030-900 Ponta Grossa, Paraná, Brazil

ARTICLE INFO

Article history:
Received 24 July 2013
Received in revised form 30 September 2013
Accepted 18 October 2013
Available online 31 October 2013

Keywords:
Antioxidant capacity
Phenolic profile
HPLC
Solvent extraction
Box–Behnken experimental design

ABSTRACT

The extraction of phenolic compounds from apples was optimised using response surface methodology (RSM). A Box–Behnken design was conducted to analyse the effects of solvent concentration (methanol or acetone), temperature and time on the extraction of total phenolic content, total flavonoids and antioxidant capacity (FRAP and DPPH). Analysis of the individual phenolics was performed by HPLC in optimal extraction conditions. The optimisation suggested that extraction with 84.5% methanol for 15 min, at 28 °C and extraction with 65% acetone for 20 min, at 10 °Cwere the best solutions for this combination of variables. RSM was shown to be an adequate approach for modelling the extraction of phenolic compounds from apples. Most of the experiments with acetone solutions extracted more bioactive compounds, and hence they had more antioxidant capacity, however, chlorogenic acid and phloridzin had higher yields (32.4% and 48.4%, respectively) in extraction with methanol.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Apples are the second most important fruit in the world (70 million tons) and are produced in temperate climate countries (Tropics of Cancer and Capricorn). They are consumed throughout the year in most countries of the world, not only for their organoleptic qualities, but also due to technological advancements in area of conservation (Braga et al., 2013).

Apples and their products contain significant amounts of phenolic compounds (Khanizadeh et al., 2008), which play an important role in maintaining human health, since they have a preventive effect against various types of diseases such as cancer, cardiovascular diseases, neuropathies and diabetes (Shahidi, 2012). Chlorogenic acid and *p*-coumaroylquinic acid are the main phenolic acids found in apples; epicatechin, catechin, procyanidins (B1 and B2), quercetins glycosides, anthocyanins and phloridzin are the major flavonoids (Khanizadeh et al., 2008; Tsao, Yang, Xie, Sockovie, & Khanizadeh, 2005). Tsao et al. (2005) reported that among the main phenols found in apples, cyanidin-3-galactoside and procyanidins have antioxidant activity three times higher and twice as high, respectively, than epicatechin and glycosides of quercitins.

There is growing interest in the study of these bioactive compounds (Kchaou, Abbès, Blecker, Attia, & Besbes, 2013; Spigno, Tramelli, & De Faveri, 2007; Wijekoon, Bhat, & Karim, 2011), and for this purpose, the first step is extracting them from the vacuolar structures and other tissues where they are found (Wink, 1997). The extraction conditions may not be the same for different plant materials since they are influenced by several parameters, such as the chemical nature of the sample, the solvent used, agitation, extraction time, solute/solvent ratio and temperature (Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012; Luthria, 2008). In addition, the oxidation of phenolic compounds should be avoided, since they are involved in the enzymatic browning reaction and consequently lose their phenol function and antioxidant capacity (Nicolas, Richard-Forget, Goupy, Amiot, & Aubert, 1994). It is advisable to use dry, frozen or lyophilised samples to avoid enzyme action (Escribano-Bailón & Santos-Buelga, 2004).

The optimisation of the extraction of phenolic compounds is essential to reach an accurate analysis. Response surface methodology (RSM) is an effective tool for optimising this process. Moreover, it is a method for developing, improving and optimising processes, and it can evaluate the effect of the variables and their interactions (Farris & Piergiovanni, 2009; Wettasinghe & Shahidi, 1999).

Thus, this study aimed to evaluate the effect of concentrations of the solvents, methanol and acetone, time and temperature on

* Corresponding author. Tel.: +55 42 32203775.
E-mail address: alessandronog@yahoo.com.br (A. Nogueira).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.10.086

the extraction of apple phenolic compounds and their antioxidant capacity using RSM as the optimisation technique.

2. Materials and methods

2.1. Materials

Gala apples (10 kg) used in the experiments were obtained in the city of Ponta Grossa (25° 05′ 42″ S 50° 09′ 43″ O), Paraná, Brazil.

The reagents Folin–Ciocalteau, Trolox (6-hydroxy-2,5,7,8-tetremethychroman-2-carboxylic acid), TPTZ (2,4,6-Tri (2-pyridyl)-s-triazine), DPPH (2,2-diphenyl-2-picrylhydrazyl), chlorogenic acid, *p*-coumaric acid, phloridzin, phloretin, (+)-catechin, (-)-epicatechin, procyanidin B1, procyanidin B2, quercetin, quercetin-3-D-galactoside, quercetin-3-β-D-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-rutinoside, caffeic acid and gallic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol, acetone, acetic acid and acetonitrile were purchased from J. T. Baker (Phillipsburg, NJ, USA) and sodium nitrite and aluminium chloride from Vetec (Rio de Janeiro, RJ, Brazil) and Fluka (St. Louis, MO, USA), respectively. The liquid nitrogen (99%) used was produced with StirLIN-1 (Stirling Cryogenics, Dwarka, New Delhi, India). The aqueous solutions were prepared using ultra-pure water (Milli-Q, Millipore, São Paulo, SP, Brazil).

2.2. Methods

2.2.1. Extraction of phenolic compounds

The apples were fragmented in a microprocessor (Metvisa, Brusque, SC, Brazil), immediately frozen with liquid nitrogen (1:2, w/v) to avoid the oxidation of the phenolic compounds (Guyot, Marnet, Sanoner, & Drilleau, 2001), and lyophilised (LD 1500, Terroni, São Paulo, SP, Brazil). The freeze-dried material (without seeds) was homogenised by crushing in a mortar. 1 g of the crushed apple was extracted with 60 mL of methanol or acetone in different concentrations, followed by incubation at different temperatures and times (Table 1). Then, the mixture was centrifuged (8160g, 20 min at 4 °C) (HIMAC CR-GII, Hitachi, Ibaraki, Japan), concentrated by evaporation under vacuum (40 °C) in a rotary evaporator (Tecnal TE-211, Piracicaba, SP, Brazil), and freeze-dried. The samples were reconstituted with 2 mL of 2.5% acetic acid and methanol (3:1, v/v) and filtered through a 0.22 μm (Nylon) syringe filter (Waters, Milford, MA, USA) prior to analysis.

2.2.2. Total phenolic content (TPC)

The total phenolic content (TPC) was determined by colorimetric analysis using Folin–Ciocalteau reagent, as described by Singleton and Rossi (1965). In a test tube, 8.4 mL of distilled water, 100 μL of sample, and 500 μL of Folin–Ciocalteau reagent were added. After 3 min, 1.0 mL of 20% sodium carbonate was added into each tube, which was agitated in a vortex (Vision Scientific CO. LTD., Korea). After 1 h, the absorbance (720 nm) was measured by spectrophotometer (model Mini UV 1240, Shimadzu, Kyoto, Japan). The measurement was compared to a calibration curve of chlorogenic acid [total phenolic concentration = 1473.3 × absorbance; $R^2 = 0.998$; $p < 0.001$] and the results were expressed as milligrams of chlorogenic acid equivalents (CAE) per kilogram of apple [mg CAE/100 g].

2.2.3. Total flavonoid content (TFC)

The total flavonoid content (TFC) of the phenolic extracts was determined using a method described by Zhishen, Mengcheng, and Jianming (1999) with modifications. 250 μL of the samples was mixed with 2.72 mL of ethanol (30%, v/v) and 120 μL of sodium nitrite solution (0.5 mol/L). After 5 min, 120 μL of aluminum chloride (0.3 mol/L) was added. The mixture was stirred and was allowed to react for 5 min. Then, 800 μL of sodium hydroxide (1 mol/L) was added and the absorbance was measured at 510 nm using a spectrophotometer (model Mini UV 1240, Shimadzu, Kyoto, Japan). The measurement was compared to a calibration curve of catechin (CT) [flavonoid concentration = 755.37 × absorbance; $R^2 = 0.996$; $p < 0.001$] and the results were expressed as milligrams of catechin equivalents (CTE) per kilogram of apple [mg CTE/100 g].

2.2.4. Measurement of in vitro antioxidant capacity

Free-radical scavenging activity of the extracts was determined in triplicate by the DPPH assay according to the Brand-Williams method, Brand-Williams, Cuvelier, and Berset (1995) with minor adaptations. This method determines the hydrogen donating capacity of molecules and does not produce oxidative chain reactions or react with free radical intermediates. Diluted samples (100 μL) were mixed with 3.9 mL of 60 μmol/L methanolic DPPH.

Table 1
Box–Behnken design applied for apple phenolic compounds extraction.

Run	Factors			
	Time (min)	Temperature (°C)	Solvent concentration (%)	
1	−1	−1	0	
2	+1	−1	0	
3	−1	+1	0	
4	+1	+1	0	
5	−1	0	−1	
6	+1	0	−1	
7	−1	0	+1	
8	+1	0	1	
9	0	−1	−1	
10	0	+1	−1	
11	0	−1	+1	
12	0	+1	+1	
13	0	0	0	
14	0	0	0	
15	0	0	0	
True values[a]			Methanol	Acetone
−1	10	10	70	50
0	15	25	85	65
+1	20	40	99.9	80

[a] Values adopted for each factor in the phenolic extraction experiment.

The absorbance was measured at 515 nm using a spectrophotometer (model Mini UV 1240, Shimadzu, Kyoto, Japan) after the solution had been allowed to stand in the dark until stabilisation (time previously determinated). Antiradical capacity was defined as the amount of apple necessary to decrease the DPPH concentration by 50%, EC_{50}. The lower the EC_{50}, the higher the antioxidant power.

The total antioxidant potential of the extracts was determined in triplicate using the ferric reducing antioxidant power (FRAP) assay as described by Benzie and Strain (1996) with minor modifications. The assay is based on the reducing power of antioxidants present in extracts, in which a potential antioxidant reduces the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}); the latter forms a blue complex (Fe^{2+}/TPTZ). Absorbance of the FRAP reagent (3 mL) was taken at 593 nm and after sample addition (100 μL); it was monitored for up to 6 min. To calculate the antioxidant capacity, the change in absorbance between the FRAP reagent and the mixture after 6 min of reaction, was correlated with a calibration curve (FRAP = 805.81 × absorbance; $R^2 = 0.999$; $p < 0.001$) of Trolox (0.1–1.0 mmol/L). The results were expressed in μmoL Trolox equivalents per kilogram of apple (μmoL TE/100 g).

2.2.5. Experimental design

In order to evaluate the extraction parameters and optimise the conditions of apple phenolic extraction, a Box and Behnken (1960) design was used. The effect of the independent variables extraction time (min), X_1, extraction temperature, X_2, and the concentration of the solvent, X_3, at three variation levels were evaluated in the extraction process (Table 1). The fifteen experiments were conducted to analyse the response pattern and to establish models for phenolic extraction, with methanol and acetone solutions separately. All experiments were carried out randomly.

A second-order polynomial equation was used to fit the experimental data of the studied variables. The generalised second-order polynomial model used in the response surface analysis is shown in Eq. (1):

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted response, β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i, and X_j are the independent variables (Bruns, Scarmino, & Barros Neto, 2006). The statistical significance of the terms in the regression equations was examined by ANOVA for each response. The terms statistically found as non-significant were excluded from the initial model and the experimental data were re-fitted only to the significant ($p \leqslant 0.05$) parameters. The simultaneous optimisation was obtained by the desirability function proposed by Derringer and Suich (1980). The optimised conditions of the independent variables were further applied to validate the model, using the same experimental procedure as made previously, in order to verify the prediction power of the models by comparing theoretical predicted data to the experimental data. Triplicate samples of the optimised proportion were prepared and analysed.

2.2.6. HPLC analysis of phenolic compounds in optimum conditions

The HPLC apparatus was a 2695 Alliance (Waters, Milford, MA, USA), with photodiode array detector PDA 2998 (Waters, Milford, MA, USA), quaternary pump and auto sampler. Separation was performed on a Symmetry C_{18} (4.6 × 150 mm, 3.5 μm) column (Waters, Milford, MA, USA) at 20 °C.

The mobile phase was composed of solvent A (2.5% acetic acid, v/v) and solvent B (acetonitrile). The following gradient was applied: 3–9% B (0–5 min), 9–16% B (5–15 min), 16–36.4% B (15–33 min), followed by an isocratic run at 100% of B (5 min) and reconditioning of the column (3% of B, 10 min). The flow rate was 1.0 mL/min. Identification of phenolic compounds was performed by comparing their retention time and spectra with those of standards. The runs were monitored at 280 nm (flavan-3-ols and dihydrochalcones), 320 nm (hydroxycinnamic acids) and 350 nm (flavonols). Quantification was performed using calibration curves of standards (at least seven concentrations were used to build the curves) (Table 2).

2.2.7. Statistical analysis

Data were presented as mean and standard deviation (SD) or pooled standard deviation (PSD). All variables had their variance analysed using the F test (two groups) orby Hartley's test ($p \geqslant 0.05$). Differences among groups were assessed by means of Student-t test for independent samples (two groups) or one-way ANOVA followed by Fisher LSD test. Pearson products (r) were used to evaluate the strength of correlation among the parameters evaluated. A p-value below 0.05 was considered significant. All statistical analyses were performed using Statistica 7.0 (StatSoft Inc., USA).

3. Results and discussion

3.1. Optimisation of extraction using methanol as solvent

The mean values of the total phenols, flavonoids, DPPH and FRAP of the extraction performed on apples with methanol are shown in Table 3. The total phenols of the methanol extraction ranged statistically ($p < 0.001$) from 457.93 (assay number 8) to 599.09 mg/100 g (central point). The highest values for total phenols were observed at the central point of the experimental design with 85.0% methanol for 15 min at 25 °C (central point).

The multiple regression analysis of total phenol values showed that the model was significant ($p < 0.001$), did not present lack of fit ($p = 0.16$) and it could explain 80.91% of all variance in data ($R^2_{adj} = 0.80$). The quadratic regression coefficient of concentration (X_3) was negative and significant. The predicted model can be described by the (Eq. 2) in terms of coded values.

$$Y = 578.93 - 80.83X_3^2 \quad (2)$$

The results suggested that time and temperature had negligible effects on the yield of total phenols.

The extraction of flavonoids ranged significantly ($p < 0.001$) from 106.81 (assay number 5) to 167.95 mg/100 g (central point). 85.0% methanol for 15 min at 25 °C were the best combination for flavonoids extraction. The model of flavonoids extraction was significant ($p < 0.001$), did not present lack of fit ($p = 0.28$) and it could explain 88.38% of variance in data (($R^2_{adj} = 0.82$). Time (X_1) significantly increased the flavonoid extraction, and quadratic regression coefficient of time (X_1), concentration(X_3) and interactions of time (X_1) and temperature (X_2); time (X_1) and concentration (X_3) had a significantly negative effect Eq. (3):

$$Y = 160.63 + 9.68X_1 - 11.68X_1^2 - 14.28X_3^2 - 11.19X_1X_2^2 - 16.35X_1X_3. \quad (3)$$

Diluted methanol (85%) was more effective in the extraction of apple phenolic compounds; it revealed that a mixture of solvents and water are more efficient than the mono-solvent system in phenolic extraction (Spigno et al., 2007). Some phenolic compounds occur naturally as glycosides (Shahidi & Naczk, 2004) and the presence of sugars makes the phenolic compounds more water soluble.

The DPPH (EC_{50}) varied significantly ($p < 0.001$) from 2008.73 (assay number 4) to 4632.13 mg/100 g (assay number 8). The high-

Table 2
Chromatographic parameters of phenolic compounds analysed by HPLC.

Phenolic compounds	Retention time (min)	UV bands (nm)	Regression equation	R^2	LOD (µg/mL)	LOQ (µg/mL)
Gallic acid	3.22	271.5	$Y = 1.27$ E + 07X + 24693	0.999	0.15	0.50
Chlorogenic acid	8.84	326.9	$Y = 1.86$ E + 07X + 877	0.997	0.19	0.62
Coumaric acid	15.09	310.7	$Y = 5.29$ E + 07X + 88036	0.999	0.03	0.09
Caffeic acid	10.56	323.8	$Y = 5$E + 07X + 39462	0.997	0.13	0.44
Catechin	8.41	278.7	$Y = 6.36$ E + 06X + 2309	0.997	0.08	0.28
Epicatechin	12.45	278.4	$Y = 5.53$ E + 06X + 161	0.997	0.07	0.23
Procyanindin B1	7.08	278.7	$y = 4.31$ E + 06X – 3176	0.997	0.54	1.81
Procyanindin B2	9.74	279.8	$Y = 4.80$ E + 06X – 2352	0.997	0.17	0.56
Phloridzin	24.12	285.5	$Y = 1$E + 07X + 37153	0.997	0.09	0.30
Phloretin	31.91	285.5	$Y = 4$E + 07X – 1E + 06	0.998	0.03	0.10
Quercetin	23.50	376.2	$Y = 1.28$ E + 06X + 9269	0.999	0.98	3.26
Quercetin-3-O-rutinoside	18.19	354.9	$Y = 2$E + 07X + 34574	0.998	0.07	0.23
Quercetin-3-O-galactoside	18.26	354.9	$Y = 4$E + 07X – 69383	0.998	0.06	0.19
Quercetin-3-O-glucoside	18.97	354.9	$Y = 2$E + 07X + 90936	0.998	0.26	0.87
Quercetin-3-O-rhamnoside	21.47	349.0	$Y = 1.56$ E + 07X + 4352	0.998	0.27	0.89
Kaempferol	28.44	364.4	$Y = 3$E + 07X + 94795	0.993	0.77	2.56
Myricetin	32.86	364.4	$Y = 2$E + 06X + 147896	0.991	0.15	0.50

Note: LOD: limit of detection; LOQ: limit of quantification.

Table 3
Total phenolic compounds (TPC), total flavonoids (TF) and antioxidant capacity by DPPH and FRAP of the extracts made with methanol solutions.

Assay	TPC (mg CAE/100 g)	TF (mg CTE/100 g)	DPPH (mg/100 g)	FRAP (µmol TE/100 g)
1	567.33[cd]	141.41[ef]	3676.77[c]	1542.18[c]
2	590.82[ab]	142.72[ef]	2638.42[f]	1665.64[b]
3	573.02[bcd]	154.21[bcde]	2567.17[f]	1565.06[c]
4	555.73[de]	146.84[cdef]	2008.73[g]	1541.70[c]
5	537.98[ef]	106.81[g]	3627.74[cd]	1570.51[c]
6	489.16[ij]	158.87[abcd]	3398.17[cde]	1554.74[c]
7	493.18[hij]	148.47[cdef]	3362.38[de]	1558.17[c]
8	457.93[k]	135.13[f]	4632.13[a]	1580.87[c]
9	475.94[jk]	139.67[f]	3615.49[cd]	1568.59[c]
10	520.75[fg]	144.44[ef]	3167.83[e]	1453.53[d]
11	500.07[hi]	145.54[def]	4153.33[b]	1450.06[d]
12	509.83[gh]	145.16[ef]	3121.51[e]	1579.33[c]
13	581.18[abc]	164.50[ab]	2453.01[f]	1853.40[a]
14	599.09[a]	160.04[abc]	2647.55[f]	1825.99[a]
15	585.36[abc]	167.95[a]	2488.10[f]	1852.02[a]
PSD[A]	46.23	15.71	510.11	132.18
p (Hartley)[B]	0.42	0.07	0.99	0.86
p (ANOVA)[C]	<0.001	<0.001	<0.001	<0.001

Note: Values expressed as mean ($n = 3$) in dry basis.
Different letters ([a, b, c, d, e, f, g, h, i, j]) in the same column represent statistical different results according to the Fischer LSD test ($p \leqslant 0.05$).
[A] PSD: pooled standard deviation.
[B] Probability values obtained by Hartley test (F max) for homogeneity of variances.
[C] Probability values obtained by One-way ANOVA.

est values for antioxidant capacity were observed in extraction with 85.0% methanol for 20 min at 45 °C. The RSM application on DPPH showed that the model was significant ($p < 0.001$), did not present lack of fit ($p = 0.24$) and could explain 97.14% of all variance in data (($R^2_{adj} = 0.94$). The temperature (X_2) significantly decreased the DPPH levels and consequently increased the antioxidant capacity. Longer times (X_1) and higher concentrations (X_3) decreased the antioxidant capacity (higher values of EC_{50}). Interations of time (X_1) and temperature (X_2) had a significantly negative effect, and time (X_1) and concentration (X_3) interations had a positive effect, according to Eq. (4):

$$Y = 2514.98 + 260.04X_1 - 402.34X_2 + 182.52X_3 + 218.72X_1^2 + 1010.48X_3^2 - 659.24X_1X_2^2 + 374.83X_1X_3. \quad (4)$$

Thoo, Ho, Liang, Ho, and Tan (2010) found similar results, where samples with better antioxidant capacity by DPPH, were obtained by extraction at 45 °C. Temperature influences the extraction, since heat renders the cell wall more permeable, enhances the solubility of the compounds, and the diffusion coeficient of the solvent. However, high temperatures (above 50 °C) can degrade some flavonoids such as antocyanins and procyanidins (Escribano-Bailón & Santos-Buelga, 2004).

The FRAP values ranged statistically ($p < 0.001$) from 1450.06 (assay number 11) to 1853.40 µM/100 g (central point). Extraction with 85.0% methanol for 15 min at 25 °C had the highest antioxidant capacity. The RSM application of FRAP values showed that the model was significant ($p < 0.001$), could explain 97.48% of all variance in data (($R^2_{adj} = 0.96$), and did not present lack of fit ($p = 0.25$). The quadatic regression coefficient of time (X_1), temperature (X_2) and concentration (X_3) was negative and significant. The interation of time (X_1) and temperature (X_2) and interation of temperature (X_2) and concentrations (X_3) had a significantly negative effect on antioxidant capacity by FRAP assay, as shown in Eq. (5):

$$Y = 1843.80 - 105.98X_1^2 - 159.18X_2^2 - 171.75X_3^2 - 36.71X_1X_2 - 61.08X_2X_3. \quad (5)$$

3.2. Optimisation of extraction using acetone as solvent

Acetone is another solvent commonly used in the extraction of phenolic compounds (Kchaou et al., 2013; Wijekoon et al., 2011). The mean values of the total phenolic content, total flavonoid content and antioxidant capacity measured by DPPH and FRAP of the extraction performed in apple with acetone solutions are shown in Table 4.

In the extracts obtained from acetone solutions, total phenols ranged statistically ($p < 0.001$) from 438.03 (assay number 6) to 778.65 mg/100 g (assay number 3). The better yields were observed in the extraction with 65% acetone at 40 °C for 10 min.

Total phenol values showed that the model was significant ($p < 0.001$), did not present lack of fit ($p = 0.15$), and could explain 96.85% of all variance in data (($R^2_{adj} = 0.94$). The concentration (X_3) significantly increased the extraction of phenolic compounds. The quadratic regression coefficient of time (X_1) and temperature (X_2) significantly increased the total phenols while the concentration (X_3) decreased. The interation between the time (X_1) and temperature (X_2) had a negative and significant effect, while interation of time (X_1) and concentration (X_3) had a positive and significant effect, as can be observed in Eq. (6):

$$Y = 580.77 + 30.01X_3 + 73.56X_1^2 + 58.09X_2^2 - 52.34X_3^2 - 58.73X_1X_2 + 122.06X_1X_3. \quad (6)$$

Total flavonoids varied statistically ($p < 0.001$) from 197.92 (assay number 12) to 333.76 mg/100 g (assay number 2). The highest values were found in the extraction with 65% acetone, for 20 min at 10 °C. The model of flavonoids was significant ($p < 0.001$), did not present lack of fit ($p = 0.20$), and it could explain 98.20% of variance in data (($R^2_{adj} = 0.96$). The temperature (X_2) and acetone concentration (X_3) significantly decreased the flavonoid levels and the quadratic regression coefficient of time (X_1) was positive and significant, whereas concentration (X_3) was negative and significant Eq. (7):

$$Y = 266.29 + 5.99X_1 - 8.84X_2 - 11.64X_3 + 34.20X_1^2 - 34.47X_3^2 - 18.34X_1^2X_2 + 22.54X_1^2X_3 - 14.01X_2X_3. \quad (7)$$

The DPPH varied significantly ($p < 0.001$) from 1615.61 (assay number 3) to 3194.00 mg/100 g (central point). Extraction with 65% acetone for10 min at 40 °C had the lowest values, but higher antioxidant capacity. The RSM application on DPPH showed that the model was significant ($p < 0.001$), did not present lack of fit ($p = 0.11$), and could explain 77.55% of all variance in data (($R^2_{adj} = 0.71$). The acetone concentration (X_3) significantly increased the DPPH levels. The quadratic regression coefficient of time (X_1) and temperature (X_2) was negative and significant, according to Eq. (8):

$$Y = 2994.92 + 248.19X_3 - 734.81X_1^2 - 495.26X_2^2 \quad (8)$$

The FRAP values ranged statistically ($p < 0.001$) from 1009.62 (assay number 6) to 2021.15 μM/100 g (assay number 2). For obtaining compounds with high antioxidant capacity, extraction with 65% acetone at 10 °C for 20 min should be performed. The RSM application of FRAP values showed that the model was significant ($p < 0.001$), did not present lack of fit ($p = 0.06$), and could explain 91.21% of all variance in data (($R^2_{adj} = 0.85$). The time (X_1) and concentration (X_3) significantly increased the FRAP levels. The quadratic regression coefficient of time (X_1) and concentration (X_3) was negative and significant, and the quadratic regression coeficient of temperature (X_2) was positive and significant. The interation of time (X_1) and concentration (X_3) had a significant effect, as shown in Eq. (9):

$$Y = 1880.04 + 135.05X_1 + 105.41X_3 - 327.96X_1^2 + 216.34X_2^2 - 227.16X_3^2 + 278.60X_1X_3. \quad (9)$$

The best yields in phenolic extraction were obtained with 65% acetone solution. This indicates that aqueous solutions are better in the phenolic extraction of apples. Other studies with fruits have had similar results, where extraction trials with 60–70% acetone were the best conditions (Kchaou et al., 2013; Wijekoon et al., 2011).

Comparison of all evaluated extractions with methanol and acetone aqueous solutions revealed that most of the acetone solutions

Table 4
Total phenolic compounds(TPC), total flavonoids (TF) and antioxidant capacity by DPPH and FRAP of the extracts made with acetone solutions.

Assay	TPC (mg CAE/100 g)	TF (mg CTE/100 g)	DPPH (mg/100 g)	FRAP (μmol TE/100 g)
1	672.39^{b}	322.78^{b}	1847.76^{f}	1655.26^{h}
2	763.72^{a}	333.76^{a}	1720.83^{fg}	2021.15^{a}
3	778.65^{a}	273.89^{d}	1615.61^{g}	1672.31^{h}
4	635.05^{c}	273.89^{d}	2096.87^{e}	1725.00^{g}
5	669.52^{b}	250.76^{h}	1761.62^{fg}	1235.90^{i}
6	438.03^{h}	258.25^{g}	1872.89^{f}	1009.62^{k}
7	521.89^{g}	261.59^{fg}	2814.17^{b}	1083.05^{j}
8	778.65^{a}	291.04^{c}	2370.06^{cd}	1971.15^{b}
9	581.63^{ef}	238.88^{i}	2488.00^{c}	1855.13^{def}
10	567.85^{f}	249.22^{h}	2182.57^{de}	1866.03^{def}
11	590.25^{e}	243.63^{i}	3012.84^{ab}	1867.31^{cdef}
12	606.33^{d}	197.92^{j}	2093.52^{e}	1888.46^{cde}
13	569.47^{f}	261.59^{fg}	3016.70^{ab}	1894.74^{cd}
14	583.12^{ef}	269.23^{de}	3194.00^{a}	1905.00^{c}
15	589.72^{e}	265.64^{ef}	2995.74^{ab}	1840.38^{def}
PSD[A]	94.13	32.14	316.90	342.85
p (Hartley)[B]	0.82	0.87	0.99	0.99
p (ANOVA)[C]	<0.001	<0.001	<0.001	<0.001

Note: Values expressed as mean (n = 3) in dry basis.
Different letters in the same column represent statistical different results according to the Fischer LSD test ($p \leqslant 0.05$).
[A] PSD: pooled standard deviation.
[B] Probability values obtained by Hartley test (*F* max) for homogeneity of variances.
[C] Probability values obtained by One-way ANOVA.

extracted more phenolic compounds than the hydro-methanolic solutions.

3.3. Verification of predictive models

The optimisation procedure was conducted in order to simultaneously maximise the total phenolic content, total flavonoids, and antioxidant capacity measured by FRAP and also to minimise DPPH values. The final result for this optimisation suggested that extraction with 84.5% methanol for 15 min, at 28 °C, and extraction with 65% acetone for 20 min, at 10 °C were the best solutions for this combination of variables. These new extractions were submitted to the same experimental analytical procedures as those applied from the beginning of this study. The observed and predicted values, along with the computed absolute errors (AE) for methanolic extraction were: total phenolics (mg/100 g) (observed: 590.82 ± 5.54; predicted: 588.81; AE = 0.34%), total flavonoids (mg/100 g) (observed: 165.55 ± 1.39; predicted: 164.47; AE = 0.66%), DPPH (mg/100 g) (observed: 2439.89 ± 72.55; predicted: 2441.10; AE = 0.05%), FRAP (μM/100 g) (observed: 1863.78 ± 24.67; predicted: 1835.31; AE = 1.55%). For extraction with the acetone solutions, the observed and predicted values, along with the computed absolute errors (AE), were: total phenolics (mg/100 g) (observed: 738.23 ± 10.52; predicted: 711.59; AE = 3.74%), total flavonoid content (mg/100 g) (observed: 334.45 ± 2.72; predicted: 325.09; AE = 2.88%), DPPH (mg/100 g) (observed: 1856.00 ± 19.90; predicted: 1958.06; AE = 5.20%), FRAP (μM/100 g) (observed: 1960.13 ± 54.43; predicted: 1934.36; AE = 1.33%).

Because of the low absolute error values obtained by the comparison between observed and predicted values, the proposed model could be used to predict the response value.

3.4. Phenolic compounds in optimum points

The phenolic profile of the extracts was determined in the best conditions of extraction for phenolic and antioxidant capacity (Table 5). The chromatograms of phenolic compounds analysed are shown in Fig. 1. Gallic, coumaric and caffeic acid, phloretin, quercetin, kaempferol and myricetin were not detected in the samples analysed by HPLC.

Except for chlorogenic acid and phloridzin, the extract from the acetone solution had the highest content ($p \leqslant 0.05$) of the individual phenols analysed. These results showed that the recovery of phenolic compounds is influenced by the polarity of the solvent used, as reported in other studies (Kchaou et al., 2013; Wijekoon et al., 2011). Methanol and acetone seem to have different specificities in the extraction of phenolic compounds.

Total phenolic compounds and total flavonoids in methanolic extractions had a significant ($p \leqslant 0.05$) correlation with antioxidant capacity measured by the DPPH ($r = -0.75$; $r = -0.52$, respectively) and FRAP ($r = 0.62$; $r = 0.53$, respectively) assays.

Flavonoids are substances with high antioxidant activity due to their redox potential. Firuzi, Lacanna, Petrucci, Marrosu, and Saso (2005) indicated that the *o*-dihydroxy structure in the B ring, the 2,3-double bond and the 3-hydroxy group in the C ring, contribute to antioxidant activity. Flavonoids also showed significant ($p < 0.01$) correlation with phloridzin contents (data not shown) in the methanolic extracts ($r = 0.90$), which agrees with the fact that this compound can be extracted to a greater extent by using methanol.

For the extracts obtained with acetone solution, the total phenolic compounds had significant ($p \leqslant 0.05$) positive correlation with flavonoids ($r = 0.52$) and consequently with catechin ($r = 0.82$), epicatechin ($r = 0.74$), procyanidins B1 ($r = 0.84$) and B2 ($r = 0.81$) (data not shown), which are the major representatives of this class. The antioxidant capacity of these extracts did not show significant ($p \geqslant 0.05$) correlation with total phenolic compounds probably due to the fact that some phenolics, extracted with acetone may display low activity with DPPH and FRAP reagents. However, among the individual phenolics analysed, only chlorogenic acid andquercetin-3-*O*-rutinosidedid not show significant ($p \geqslant 0.05$) correlation with antioxidant capacity by DPPH assay. Chlorogenic acid has very low activity in FRAP assay, as demonstrated by Tsao et al. (2005). This could explain the fact that it did not have a correlation with antioxidant capacity in extraction by methanol or acetone.

Other studies have revealed that methanolic solutions are more effective for catechin extraction (Escribano-Bailón & Santos-Buelga, 2004; Tabart et al., 2007), however, in the present study better yields were obtained with acetone, as well as a good correlation with total phenolic content ($r = 0.82$, p = 0.02).

The procyanidins B1 and B2 are the compounds that showed the highest difference in content between the extractions with methanol and acetone, being approximately 35% higher. Foo and Porter (1981) have reported that acetone solutions gave higher yields with highly polymerised flavanoids from fruits. Santos-Buelga and Scalbert (2000) have reported that the high antioxidant capacity of procyanindins is due to the presence of the catechol unit on the aromatic B-ring, which stabilises the free radicals and their ability to chelate metals and proteins due to several *o*-dihydroxy phenolic groups in their high molecular weight structure. This could explain the higher antioxidant capacity of acetone extracts and the good correlations ($p < 0.03$) of procyanidins B1 and B2 with the DPPH ($r = 0.81$; $r = 0.71$, respectively) and FRAP ($r = 0.79$; $r = 0.56$, respectively) assays.

Table 5
Phenolic profile of applesin the optimum conditions of solvents extraction.

Compounds	Solvent		*p*-Value[A]	*p*-Value[B]
	Methanol	Acetone		
Chlorogenic acid	55.34 ± 0.10^{a}	41.79 ± 0.11^{b}	0.89	<0.001
Catechin	9.67 ± 0.13^{b}	10.54 ± 0.22^{a}	0.17	0.02
Epicatechin	25.56 ± 0.09^{b}	26.86 ± 0.29^{a}	0.07	0.01
Procyanidin B1	21.87 ± 0.14^{b}	29.68 ± 0.36^{a}	0.27	<0.001
Procyanidin B2	26.88 ± 0.11^{b}	39.53 ± 0.29^{a}	0.25	<0.001
Phloridzin	1.66 ± 0.05^{a}	11.22 ± 0.05^{b}	0.20	<0.001
Quercetin-3-O-rutinoside	6.77 ± 0.03^{b}	7.01 ± 0.08^{a}	0.24	0.01
Quercetin-3-O-galactoside	8.15 ± 0.02^{b}	8.76 ± 0.06^{a}	0.27	<0.001
Quercetin-3-O-glucoside	2.14 ± 0.01^{b}	2.24 ± 0.01^{a}	0.92	<0.001
Quercetin-3-O-rhamnoside	3.33 ± 0.02^{b}	4.00 ± 0.04^{a}	0.29	<0.001

Different letters ($^{a,\ b}$) in the same line represent statistically different results ($p \leqslant 0.05$).
[A] Probability values obtained by *F* test for homogeneity of variances.
[B] Probability values obtained by *T* test.

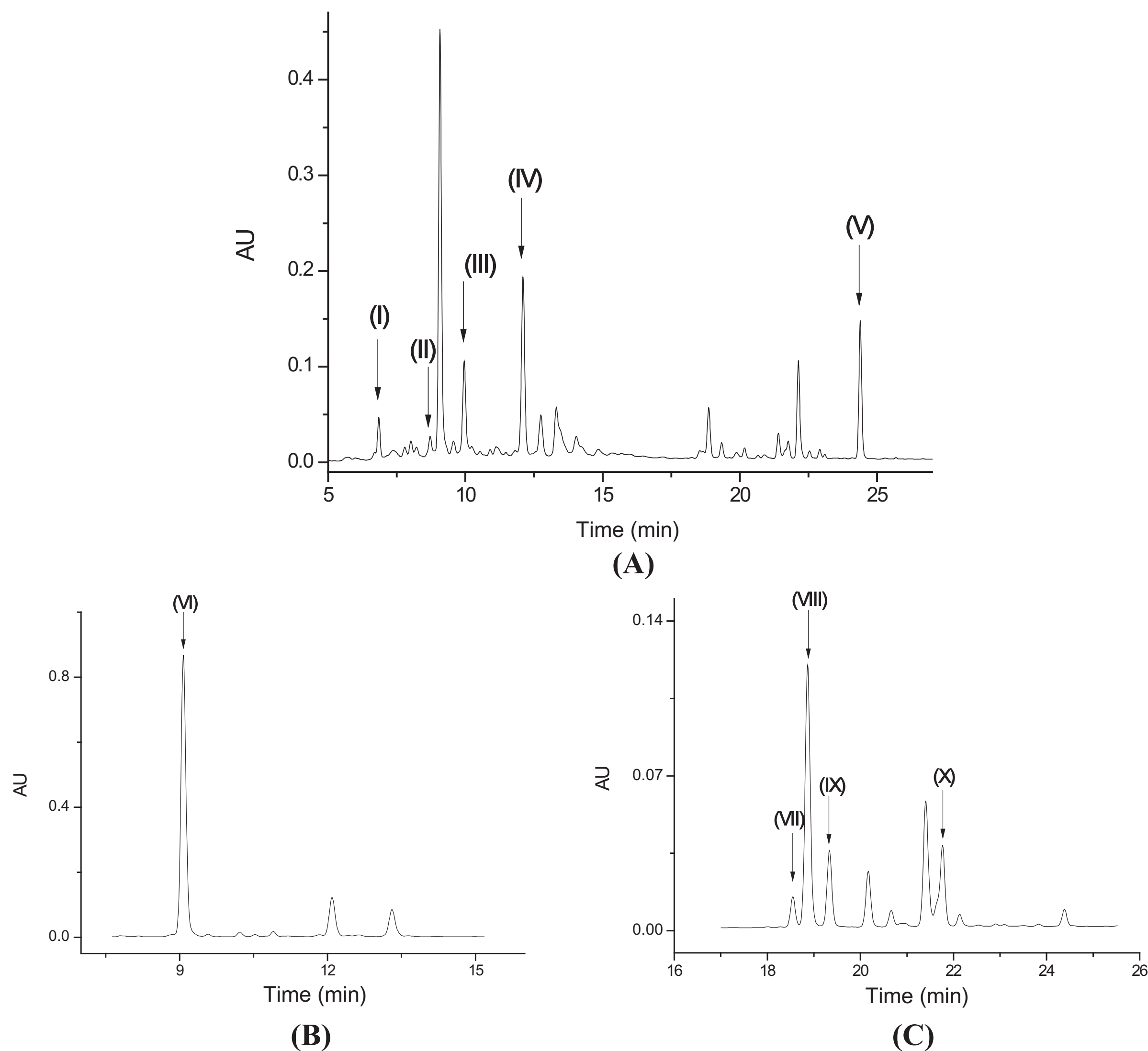

Fig. 1. Chromatograms obtained of phenolic extract of apple at 280 ηm (A), 320 ηm (B) and 350 ηm (C). (I) procyanindin B1, (II) catechin, (III) procyanindin B2, (IV) epicatechin, (V) phloridzin, (VI) chlorogenic acid, (VII) quercetin-3-O-rutinoside, (VIII) quercetin-3-O-galactoside, (IX) quercetin-3-O-glucoside and (X) quercetin-3-O-rhamnoside.

In fact, solvents with different polarities may be required to extract more phenolic contents. For reach better yields, a sequential extraction with methanol and acetone solutions might be done. The optimal conditions achieved in this study can be useful to research procedures with apple phenolic compounds. However, the use of solvents such as methanol and acetone are not recommended if the intent is to obtain extracts for use in food and drugs, due to its toxicity. For this purpose, the extraction should be done using other solvents, although not be achieved the same yields (data not shown).

4. Conclusion

RSM was effective in estimating the effect of three independent variables on the extraction of total phenolic compounds in apples, as well as total flavonoids and antioxidant capacity measured by DPPH and FRAP. The best combinations of the variables for increasing the yield of total phenolic content, total flavonoid compounds and antioxidant capacity was obtained with 84.5% methanol for 15 min, at 28 °C and extraction with 65% acetone for 20 min, at 10 °C. In optimal conditions, methanol extracted more chlorogenic acid and phloridzin than acetone, while catechin, epicatechin, procyanidins (B1 and B2) and glycosides of quercitin were extracted to a greater extent with acetone.

Acknowledgements

The authors are deeply grateful to the Coordination for the Improvement of Personnel in Higher Level (CAPES), National Council of Scientific and Technological Development (CNPq) and the Araucaria Foundation (FA) for support and scholarships.

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.2013.10.086.

References

Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry, 239*(1), 70–76.

Box, G. E. P., & Behnken, D. W. (1960). Some new three level designs for the study of quantitative variables. *Technometrics, 2*(4), 455–475.

Braga, C. M., Zielinski, A. A. F., Silva, K. M., Souza, F. K. F., Pietrowski, G. A. M., Couto, M., Granato, D., Wosiacki, G., & Nogueira, A. (2013). Classification of juices and fermented beverages made from unripe, ripe and senescent apples based on the aromatic profile using chemometrics. *Food Chemistry, 141*(2), 967–974.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT – Food Science and Technology, 28*(1), 25–30.

Bruns, R. E., Scarmino, I. S., & Barros Neto, B. (2006). *Statistical design – chemometrics*. Amsterdam: Elsevier, p. 273.

Derringer, G., & Suich, R. (1980). Simultaneous optimization of several response variables. *Journal of Quality Technology, 12*, 214–219.

Escribano-Bailón, M. T., & Santos-Buelga, C. (2004). Polyphenol extraction from foods. In C. Santos-Buelga & G. Williamson (Eds.), *Methods in polyphenol analysis* (pp. 1–16). Cambridge: Royal Society of Chemistry.

Farris, S., & Piergiovanni, L. (2009). Optimization of manufacture of almond paste cookies using response surface methodology. *Journal of Food Process Engineering, 32*(1), 64–87.

Firuzi, O., Lacanna, A., Petrucci, R., Marrosu, G., & Saso, L. (2005). Evaluation of the antioxidant activity of flavonoids by "ferric reducing antioxidant power" assay and cyclic voltammetry. *Biochimica et Biophysica Acta, 1721*(1–3), 174–184.

Foo, L. Y., & Porter, L. J. (1981). The structure of tannins of some edible fruits. *Journal of the Science of Food and Agriculture, 32*, 711–716.

Guyot, S., Marnet, N., Sanoner, P., & Drilleau, J.-F. (2001). Direct thiolysis on crude apple materials for high-performance liquid chromatography characterization and quantification of polyphenols in cider apple tissues and juices. *Methods in Enzymology, 335*, 57–70.

Haminiuk, C. W. I., Maciel, G. M., Plata-Oviedo, M. S. V., & Peralta, R. M. (2012). Phenolic compounds in fruits – An overview. *International Journal of Food Science & Technology, 47*(10), 2023–2044.

Kchaou, W., Abbès, F., Blecker, C., Attia, H., & Besbes, S. (2013). Effects of extraction solvents on phenolic contents and antioxidant activities of Tunisian date varieties (*Phoenix dactylifera L.*). *Industrial Crops and Products, 45*, 262–269.

Khanizadeh, S., Tsao, R., Rekika, D., Yang, R., Charles, M. T., & Rupasinghe, H. P. V. (2008). Polyphenol composition and total antioxidant capacity of selected apple genotypes for processing. *Journal of Food Composition and Analysis, 21*(5), 396–401.

Luthria, D. L. (2008). Influence of experimental conditions on the extraction of phenolic compounds from parsley (*Petroselinumcrispum*) flakes using a pressurized liquid extractor. *Food Chemistry, 107*(2), 745–752.

Nicolas, J. J., Richard-Forget, F. C., Goupy, P. M., Amiot, M. J., & Aubert, S. Y. (1994). Enzymatic browning reactions in apple and apple products. *Critical Reviews in Food Science and Nutrition, 34*(2), 109–157.

Santos-Buelga, C., & Scalbert, A. (2000). Proanthocyanidins and tannin-like compounds – nature, occurrence, dietary intake and effects on nutrition and health. *Journal of the Science of Food & Agriculture, 80*(7), 1094–1117.

Shahidi, F., & Naczk, M. (2004). *Phenolics in food and nutraceuticals*. Florida: CRC Press, p. 558.

Shahidi, F. (2012). Nutraceuticals, functional foods and dietary supplements in health and disease. *Journal of Food & Drug Analysis, 20*, 226–230.

Singleton, V., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144–158.

Spigno, G., Tramelli, L., & De Faveri, D. M. (2007). Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *Journal of Food Engineering, 81*(1), 200–208.

Tabart, J., Kevers, C., Sipel, A., Pincemail, J., Defraigne, J.-O., & Dommes, J. (2007). Optimisation of extraction of phenolics and antioxidants from black currant leaves and buds and of stability during storage. *Food Chemistry, 105*(3), 1268–1275.

Thoo, Y. Y., Ho, S. K., Liang, J. Y., Ho, C. W., & Tan, C. P. (2010). Effects of binary solvent extraction system, extraction time and extraction temperature on phenolic antioxidants and antioxidant capacity from mengkudu (*Morindacitrifolia*). *Food Chemistry, 120*(1), 290–295.

Tsao, R., Yang, R., Xie, S., Sockovie, E., & Khanizadeh, S. (2005). Which polyphenolic compounds contribute to the total antioxidant activities of apple? *Journal of Agricultural and Food Chemistry, 53*(12), 4989–4995.

Wettasinghe, M., & Shahidi, F. (1999). Evening primrose meal: A source of natural antioxidants and scavenger of hydrogen peroxide and oxygen-derived free radicals. *Journal of Agricultural and Food Chemistry, 47*(5), 1801–1812.

Wijekoon, M. M. J. O., Bhat, R., & Karim, A. A. (2011). Effect of extraction solvents on the phenolic compounds and antioxidant activities of bungakantan (*Etlingeraelatior* Jack) inflorescence. *Journal of Food Composition and Analysis, 24*(4–5), 615–619.

Wink, M. (1997). Compartmentation of secondary metabolites and xenobiotics in plant vacuoles. *Advances in Botanical Research, 25*, 141–169.

Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry, 64*(4), 555–559.