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The Optimization of Extraction of Antioxidants from Apple Pomace by Pressurized Liquids

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Pressurized liquid extraction (PLE) is a green extraction technique that can enhance extraction rates of bioactive compounds. PLE was used to extract antioxidants and polyphenols from industrially generated apple pomace at two different temperature ranges: 160 to 193 °C and 75 to 125 °C. Antioxidant activity (DPPH radical scavenging test), total phenol content and three individual polyphenol groups were determined. Response surface methodology was used to optimize the five response values. Maximum antioxidant activity was obtained at a temperature of 200 °C, but unwanted compounds such as hydroxymethylfurfural were formed. Therefore a lower temperature range between 75 and 125 °C is recommended. Using this temperature range, a maximum antioxidant activity was determined at 60% ethanol and 102 °C. By using PLE the antioxidant activity was increased 2.4 times in comparison to traditional solid−liquid extraction, and the technique may be a promising alternative to conventional techniques for extracting antioxidants.

KEYWORDS: Accelerated solvent extraction; response surface methodology; polyphenols; HPLC; DPPH

INTRODUCTION

Sustainable food production and waste valorization are becoming important issues in the food industry. Food processors generate high amounts of biological byproduct and waste that could be used for other purposes. Apples and apple products are consumed and processed in high quantities in various countries all over the world. Several million tonnes of apple pomace, which consists of the leftovers after apple pressing for the production of apple juice and apple cider, is currently generated (*1*). Apple pomace has been utilized as a source for several applications (*2*), including pectin recovery (*3*), animal feed (*4*, *5*), citric acid production (*6*), ethanol production (*7*) and enzyme production (*8*, *9*). Another important application is the recovery of polyphenols as bioactive compounds (*1*). Individual polyphenolic compounds present in apple pomace have potential health benefits, since they show anticarcinogenic activity *in vitro* (*10*). In addition, recently McCann et al. (*11*) have shown that crude extracts from apple pomace can prevent colon cancer *in vitro*. The most abundant polyphenols present in apples are chlorogenic acid, phloretin glucosides and quercetin glucosides (*12*−*14*). Other polyphenolic compounds such as catechins and procyanidins have also been identified, but are present in relatively small amounts (*15*).

While the potential of apple pomace as a source of polyphenols seems clear, there is less information on potential of strategies for the recovery of these compounds from the intact source. One way of increasing extraction rates of bioactive compounds from food matrices is by using pressurized liquid extraction (PLE). This technique uses high pressures, allowing the user to carry out extractions at temperatures above the boiling point of the solvent (*16*). This enhances analyte solubility and mass transfer rates, resulting in better recoveries of the target compounds than conventional solid−liquid extraction techniques. The high temperatures used in PLE also decrease the viscosity and the surface tension of the solvents. This means that the sample matrix area is accessed more easily, which also improves the extraction rate. In this way PLE uses a combination of high pressures and temperatures that provide faster extraction processes which require small amounts of solvents. PLE also generally requires smaller amounts of organic solvents than conventional techniques and could therefore be considered a green extraction technique (*17*).

To date PLE has mainly been used at an analytical level for the quantitative recovery of target analytes. For instance, PLE has been used to optimize analytical extraction of polyphenols from apple using methanol as an organic solvent (*18*). The technique has also been used to optimize the sample preparation procedure for analyzing polyphenols from parsley (*19*). In recent times the technique has shown potential as a green extraction method to obtain crude extracts with useful biological properties. For example, PLE was used to optimize the extraction of antioxidants from microalgae (*20*), anthocyanins from dried red grape skin (*21*), antioxidants from rosemary (*22*), and vitamin E rich oil was isolated from grape seeds using PLE (*23*). A number of variables can be optimized to maximize yields of a target compound using PLE. For example, solvent composition can be varied and theoretically temperatures up to critical points of applied solvents can be used. Therefore response surface methodology (RSM) could be a useful tool for optimizing extractions where a number of variables can be optimized. RSM is a combination of mathematical and statistical techniques, in which modeling is used to optimize a response value that is influenced by

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various factors. RSM has been widely used in various fields to optimize processing conditions, and one of these applications is to extract bioactive compounds from fruit and vegetable sources (*20*, *24*, *25*). In a previous study the polyphenol composition from apple pomace obtained from the Irish apple cider industry was shown to have potential as an antioxidant ingredient (*14*). The objective of the present study was to optimize PLE of industrially produced apple pomace using ethanol and water, two environmentally and food safe solvents, with the aim of maximizing antioxidant and polyphenol levels. RSM was used to minimize the number experiments required to identify optimal conditions.

MATERIALS AND METHODS

Chemicals. Procyanidins A1, B1, and B2 were purchased from Extrasynthèse (Lyon, France). Caffeic acid, (+)-catechin, chlorogenic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), epicatechin, ferulic acid, Folin−Ciocalteu Reagent (FCR), 5-(hydroxymethyl)furfural (HMF), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), *p*-coumaric acid, phloridzin, rutin, and *trans*-4-hydroxy-3-methoxy cinnamic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Preparation of Materials. Apple pomace was provided by Bulmers Limited (Clonmel, Ireland). Bulmers Limited uses a blend of cider apple varieties. On arrival the samples were vacuum packed and stored at −20 °C for at least 24 h. Following this, samples were removed from the vacuum pack and lyophilized for a minimum of 5 days in an A6/14 freeze-dryer (Frozen in Time Ltd., York, England). The lyophilized samples were then vacuum packed and stored at −20 °C until required for analysis.

PLE of Antioxidants and Polyphenols. On the day of extraction, lyophilized samples were milled to a fine powder using a blender (BL440001, Kenwood Limited, Hampshire, England). The particle size distribution of the apple pomace powder was as follows: 7.5% > 1.4 mm; 10.9% > 850 μm and < 1.4 mm; 10.3% > 600 μm and < 800 μm; 71.3% < 600 μm. The moisture content of the apple pomace powder was 5.24%. To extract the sample by PLE an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA) was used. To avoid clogging 1 g of apple pomace was added between two layers of diatomaceous earth in the 22 mL extraction cell. A cellulose filter was used in the bottom of the cell. Different temperature ranges and ethanol:water concentrations were used in order to identify optimal conditions for antioxidant and polyphenol extraction by RSM. A standard stepwise PLE extraction protocol was used for all extractions. (1) The PLE cell was loaded into the extraction compartment and heated up to the temperature specified by the RSM design (X_1 °C). (2) The cell was filled with ethanol and water concentration at the specified concentration (X_2 %) until a pressure of 10.3 MPa was reached. (3) A static extraction of 5 min when pressure and temperatures reached were maintained was then applied. (4) The cell was rinsed by passing an additional 60% of the volume of the solvent mixture which had already crossed the cell. Extracts were collected in 60 mL glass vials (Dionex, Sunnyvale, CA), and their volume was measured. In all methods described below the final volume and therefore concentration of apple pomace was taken into account in calculations. Prior to analysis the samples were centrifuged for 15 min at 2238*g* (MSE Mistral 3000i, Sanyo Gallenkamp, Leicestershire, England). Ten milliliters Of the supernatant was filtered through 0.22 μm PVDF syringe filters (Pall Gelman Laboratory, Portsmouth, England). The extracts were stored at −20 °C.

DPPH Assay. A modified version of the DPPH method (*26*) was used. Trolox was used as a standard in the DPPH assay. Standard samples were prepared by diluting a methanolic Trolox stock solution (0.2 mM). The Trolox standard samples and blanks were used to make a calibration curve. A working DPPH solution (0.048 mg/mL) was prepared by making a 1 in 5 dilution of the methanolic DPPH stock solution (0.238 mg/mL). Prior to analysis, serial dilutions of the ethanolic extracts of the apple pomace samples were prepared. 500 μL of diluted ethanolic extracts was added to 500 μL of the DPPH working solution into microcentrifuge tubes. After vortexing, the tubes were incubated at room temperature for 30 min. The absorbance was then measured against ethanol at 515 nm in 1 mL propylene cuvettes using a spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Kyoto, Japan). The decrease in absorbance of a sample was calculated in comparison to a blank sample (500 μL of methanol and 500 μL of DPPH). The relative decrease in absorbance (PI) was then calculated as follows: PI (%) = $1 - (A_e/A_b)$, with A_e = absorbance of sample extract and A_b = absorbance of blank. The PIs used to calculate the related antioxidant activity were superior (PI_1) and inferior (PI_2) to the value estimated at 50%. The antioxidant activity was defined as the concentration of sample extract necessary to obtain an activity of 50% (IC_{50}). In all experiments the IC_{50} of Trolox was determined as well. The final results for antioxidant activity were determined by using the following equation: antioxidant activity = $(IC_{50Trolox}/IC_{50Sample}) \times 10^5$ (*27*). The antioxidant activity was expressed in mg Trolox equivalent (TE) per 100 g dry weight apple pomace (mg TE/100 g DW).

FCR Assay. Total phenolic content of apple pomace extracts was assessed using a modified version of the Folin−Ciocalteu assay (*28*). Gallic acid was used as a standard, and the aqueous gallic acid solution (200 mg/L) was diluted with distilled water to give appropriate concentrations for a standard curve. For the analysis, 100 μL of sample extract or gallic acid standard, 100 μL of methanol, 100 μL of Folin−Ciocalteu reagent and 700 μL of Na_2CO_3 were added into a 1.5 mL microcentrifuge tube. The samples were vortexed immediately, and the tubes were incubated in the dark for 20 min at room temperature. After incubation all samples were centrifuged at 15682*g* for 3 min. The absorbance of the supernatant was then measured at 735 nm in 1 mL plastic cuvettes using a spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Kyoto, Japan). The results were expressed in mg gallic acid equivalent/100 g dry weight apple pomace (mg GAE/100 g DW).

Polyphenol Analysis by HPLC-DAD. HPLC analysis was performed on a Shimadzu SCL 10A chromatography system (Shimadzu, Kyoto, Japan), equipped with an LC-10 AT VP pump, a CTO-10 AC column thermostat, a SIL 10 AD autosampler and an SPD-M10A diode array detector with an absorbance detection range between 190 and 800 nm. Separations were conducted on a Zorbax SB C_{18}, 5 μm, 150 × 4.6 mm column (Agilent Technologies, Santa Clara, CA). The gradient profile was based on a method of Tsao and Yang (*29*). Acetic acid in 2 mM sodium acetate (final pH 2.55, v/v) was used as eluent A and 100% acetonitrile was used as eluent B. The column temperature was set at 37 °C, and the flow rate was 1 mL/min. The solvent gradient program was set as follows: initial conditions 95% A, 5% B; 0−45 min, 5−15% B; 45−60 min, 15−30% B; 60−65 min, 30−50% B; 65−70 min, 50−100% B. Prior to injection sample extracts were filtered through PVDF syringe 0.22 μm filters. The injection volume was 10 μL. As recommended by Tsao and Yang (*29*) hydroxybenzoic acids, dihydrochalcones, procyanidins and flavanols were monitored at a wavelength of 280 nm, hydroxycinnamic acid derivatives at 320 nm, and flavonols at 360 nm. For quantification and identification purposes external calibration curves were prepared of the standards listed above. The levels of chlorogenic acid were expressed in μg chlorogenic acid/g DW apple pomace, the total flavonol level in μg rutin/g DW apple pomace, the level of phloretin glycosides in μg phloridzin/g DW apple pomace. In addition to polyphenols, hydroxymethylfurfural could be detected with the above-mentioned method, based on its spectrum and retention time. HMF was quantified at 280 nm and the level was expressed in mg/g DW apple pomace.

Melanoidins. To estimate the degree of formation of browning compounds such as melanoidins the absorbance at 420 nm was measured; this wavelength has previously been used to detect browning defects in fruit juices (*30*).

Experimental Design. RSM was used to optimize antioxidant extraction from apple pomace. A Central Composite design with two independent variables was used: ethanol concentration (X_1) and temperature (X_2). The preliminary RSM design used was with a broad temperature range: 80−170 °C. From the preliminary design, it was decided to carry out two RSM designs with two different temperature ranges. The first RSM design was focused on a higher temperature range of 160 (coded as −1) to 193 °C (coded as +1), and the temperature of the third RSM design was to use a milder temperature ranging from 75 (coded as −1) to 125 °C (coded as +1). The actual and corresponding values of the designs are shown in **Tables 1** and **2**. The two independent variables were coded at five levels ($-\alpha$, −1, 0, 1, α), which resulted in an experimental design of 13 experimental points, including five central points. Design Expert 7.1.3 (Stat-Ease Inc., Minneapolis, MN) was used for designing experiments and statistical data analysis (ANOVA).

Table 1. Response Surface Design and Corresponding Response Values of Pressurized Liquid Extraction in a Temperature Range of 160 to 193 °C[a]

run no.	X_1	X_2	response 1, DPPH	response 2, FCR	response 3, CHA	response 4, FLA	response 5, PHLOR	response 6, absorbance at 420 nm
1	50 (0)	176.50 (0)	2619 ± 20	4889 ± 34	651	912	672	2.136
2	50 (0)	176.50 (0)	2621 ± 15	4667 ± 35	639	945	670	2.078
3	85.36 (+α)	176.50 (0)	853 ± 9	3157 ± 24	539	937	956[f]	2.012
4	50 (0)	176.50 (0)	2792 ± 12	4592 ± 42	681	951	689	2.464
5	50 (0)	176.50 (0)	2800 ± 71	4595 ± 11	694	1054	664	2.252
6	75 (1)	193 (1)	2627 ± 44	5072 ± 72	719	891	652	4.685[g]
7	50 (0)	153 (−α)	1281 ± 19	3054 ± 35	301	1025	646	0.811
8	50 (0)	176.50 (0)	2680 ± 16	4600 ± 29	680	974	668	2.21
9	25 (−1)	193 (1)	3309 ± 36	4993 ± 18	500	431	258	2.735
10	14.64 (-α)	176.50 (0)	2347 ± 13	4119 ± 14	720[d]	1047[e]	663[f]	1.102
11	50 (0)	200 (+α)	4428[b] ± 38	5782[c] ± 86	733	970	666	3.735
12	75 (1)	160 (−1)	1076 ± 23	2677 ± 41	330	1117	863	1.007
13	25 (−1)	160 (−1)	1489 ± 32	3026 ± 27	246	295	150	0.974

[a] X_1 = ethanol concentration (%), X_2 = temperature (°C); DPPH = antioxidant activity (mg TE/100 g DW); FCR = phenol level (mg GAE/100 g DW); CHA = level of chlorogenic acid (μg chlorogenic acid/g DW); FLA = sum of level of flavonols (μg rutin/g DW); PHLOR = level of phloretin glycoside (μg phloridzin/g DW) and the level of absorbance at 420 nm. [b] This value was considered an outlier and was not taken into consideration when describing the model for antioxidant activity. [c] This value was considered an outlier and was not taken into consideration when describing the model for phenol levels by FCR. [d] This value was considered an outlier and was not taken into consideration when describing the model for the level of chlorogenic acid. [e] This value was considered an outlier and was not taken into consideration when describing the model for the level of flavonols. [f] This value was considered an outlier and was not taken into consideration when describing the model for the level of phloretin glycosides. [g] This value was considered an outlier and was not taken into consideration when describing the model for the level of absorbance at 420 nm.

Table 2. Response Surface Design and Corresponding Response Values of Pressurized Liquid Extraction in a Temperature Range of 75 to 125 °C[a]

run no.	X_1	X_2	response 1, DPPH	response 2, FCR	response 3, CHA	response 4, FLA	response 5, PHLOR
1	50 (0)	100 (0)	902 ± 12	1473 ± 12	537	1093	739
2	50 (0)	100 (0)	1013 ± 14	1476 ± 24	571	1232	845
3	50 (0)	100 (0)	1028 ± 25	1457 ± 14	548	1265	848
4	50 (0)	135.36 (+α)	393 ± 8	565[c] ± 9	243[d]	489	389
5	50 (0)	64.64 (−α)	550 ± 5	1195 ± 12	457[d]	1166[e]	668[f]
6	85.36 (+α)	100 (0)	707 ± 15	1445 ± 26	520	1258	904
7	14.64 (−α)	100 (0)	448[b] ± 4	1036 ± 8	431	1262	684
8	25 (−1)	125 (1)	1132[b] ± 58	1698 ± 7	526	1208	741
9	75 (1)	125 (1)	748 ± 18	1006 ± 15	361	765	500
10	50 (0)	100 (0)	952 ± 41	1414 ± 3	535	1171	829
11	25 (−1)	75 (−1)	344 ± 1	519 ± 9	199	375	265
12	75 (1)	75 (−1)	424 ± 20	1173[c] ± 6	468	1152	724
13	50 (0)	100 (0)	964 ± 18	1470 ± 30	529	1262	778

[a] X_1 = ethanol concentraion (%), X_2 = temperature (°C); DPPH = antioxidant activity (mg TE/100 g DW); FCR = phenol level (mg GAE/100 g DW); CHA = level of chlorogenic acid (μg chlorogenic acid/g DW); FLA = sum of level of flavonols (μg rutin/g DW); PHLOR = level of phloretin glycoside (μg phloridzin/g DW). [b] This value was considered an outlier and was not taken into consideration when describing the model for antioxidant activity. [c] This value was considered an outlier and was not taken into consideration when describing the model for phenol levels by FCR. [d] This value was considered an outlier and was not taken into consideration when describing the model for the level of chlorogenic acid. [e] This value was considered an outlier and was not taken into consideration when describing the model for the level of flavonols. [f] This value was considered an outlier and was not taken into consideration when describing the model for the level of phloretin glycosides.

Data Analysis. Data were analyzed to fit a second order polynomial model (eq 1).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{12} X_1 X_2 \quad (1)$$

where Y = response value, β = constant coefficients, and X = independent variable.

When needed, outliers were identified and were not taken into consideration when describing the relevant models (**Table 1** and **2**). The models were predicted through statistical analysis and regression analysis (ANOVA) using Design Expert 7.1.3.

RESULTS AND DISCUSSION

A preliminary PLE using a broad temperature range indicated that two RSM experiments were required to optimize extractions. The first experiment used a high temperature range (160−193 °C), to further optimize high antioxidant activities detected at the higher temperature range in the preliminary experiment. Since browning of the extracts in this temperature range was apparent, another milder temperature regime was investigated, ranging from 75 to 125 °C. The results of the two experiments are discussed below.

High Temperature Range (160 to 193 °C). In this temperature range six response values were analyzed: antioxidant activity as measured by DPPH, phenol content as measured by FCR, chlorogenic acid level, the sum of flavonol levels, phloretin glycoside and melanoidins. In a previous study optimal conditions were determined by preparing apple pomace extracts by ethanolic solid−liquid extractions (*31*). The main polyphenols extracted were chlorogenic acid, flavonols, and phloretin glucosides. In this study, these three polyphenol groups were also the main polyphenol groups detected when apple pomace was extracted with PLE and ethanol/water as solvents. Catechins and procyanidins and other cinnamic acids such as caffeic acid and *p*-coumaric acid were only present in very small amounts. This is in agreement with other authors, who found similar major polyphenolic compounds when extracting apple pomace with 100% ethanol (*12*). Anthocyanins were not detected; anthocyanins are only detected in red apple varieties (*32*). The apples Bulmers Ltd. uses for cider production are a blend of varieties, both red and green. Anthocyanins are unstable compounds (*33*) and if at all present may have oxidized during apple pomace collection or processing.

Table 3. Regression Coefficients of Significant Terms, the Coefficient of Determination (R^2), Standard Deviations (SD) and Lack of Fit Values of the Optimized Second Order Polynomial Models of Antioxidant Activity as Measured by DPPH (mg TE/100 g DW), Phenol Content (mg GAE/100 g DW), Level of Chlorogenic Acid (μg Chlorogenic Acid/g DW), Flavonol Level (μg Rutin/g DW) and Phloretin Glycoside Level (μg Phloridzin/g DW) for Pressurized Liquid Extracts of Apple Pomace in a Temperature Range of 160 to 193 °C

	predicted value					
constant coefficient	DPPH	FCR	chlorogenic acid	flavonols	phloretin glycoside	melanoidins
intercept β_0	2673	4616	669	971	668	2.25
concn β_1	−401	−204	82	333	277	0.22
temp β_2	890	1095	157			1.00
concn × temp β_{12}			34	−91	−80	
concn × concn β_{11}	−540	−551	−131	−270	−187	−0.33
temp × temp β_{22}			−80			
R^2	0.97	0.96	0.99	0.97	0.99	0.97
residual SD	162	213	23	49	23	0.2
residual SD as % mean	7.35	5.18	4.08	5.64	3.79	8.70
P value of lack of fit test	0.06	0.08	0.46	0.63	0.02	0.32

Response values for each set of variable combinations for PLE samples at the high temperature range are presented in **Table 1**. Statistical analyses by ANOVA indicated that all response values (antioxidant activity by DPPH, phenol content by FCR, chlorogenic acid level, level of the sum of flavonols, level of phloretin glucosides and melanoidins) fitted best to the second order polynomial model, and this model was used subsequently. **Table 3** describes regression coefficients and statistics of the resulting models of PLE ethanolic extraction from 160 to 193 °C. One outlier had to be removed from the responses for antioxidant activity, phenol content, level of chlorogenic acid, summed level of flavonols and absorbance at 420 nm in order to obtain valid models (**Table 1**).

All models for the PLE at this temperature range were found to be significant (p-value < 0.05) and the lack of fit nonsignificant (p-value > 0.05). Only the lack of fit of the response for the level of phloretin glycoside was still significant (p-value $= 0.02$) even after removing two outliers from the response (**Tables 1** and **3**). The models for all response values were highly adequate, since the coefficient of determination (R^2) of these responses was greater than 0.96 (**Table 3**). All second order polynomial response values were significantly affected by ethanol concentration (β_1) and the quadratic factor of ethanol concentration (β_{11}).

The antioxidant activity as measured by DPPH was significantly affected by temperature. As can be seen from **Figure 1a**, the DPPH values increased with increasing temperature. Chlorogenic acid was affected by both ethanol concentration and temperature (**Table 1** and **Figure 1b**).

Solvent concentration played the most important role in the extraction of polyphenols. Due to the smaller size and the presence of carboxylic groups, chlorogenic acid is more water-soluble than flavonols and phloretin glycosides, which leads to a different optimum in ethanol concentration. In addition, chlorogenic acid can be noncovalently bound to melanoidins a product of Maillard reactions and/or caramelization. Degree of absorbance at 420 nm is generally used in fruit juices to give an estimation of the melanoidin level (*30*). In the present study absorbance values at 420 nm for PLE extracts were found to increase with increasing temperature (**Figure 1c**). In fact the model for absorbance at 420 nm (and hence melanoidins) showed a very similar RSM surface plot to the antioxidant activity

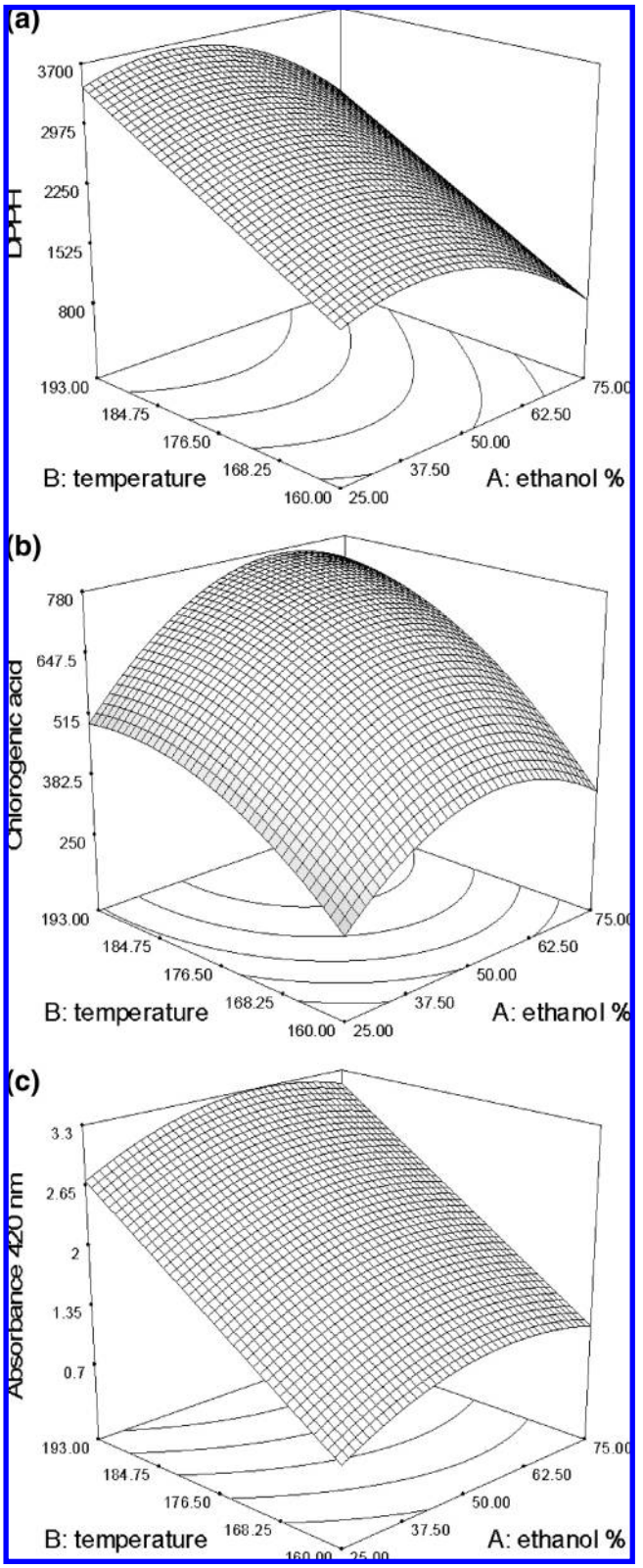

Figure 1. Response surface plot of PLE between 160 and 193 °C showing the effects of temperature and ethanol concentration on (**a**) antioxidant activity as measured by DPPH (mg TE/100 g DW apple pomace), (**b**) the level of chlorogenic acid (μg chlorogenic acid/g DW apple pomace), and (**c**) the absorbance at 420 nm.

Table 4. Regression Coefficients of Significant Terms, the Coefficient of Determination (R^2), Standard Deviations (SD) and Lack of Fit Values of the Optimized Second Order Polynomial Models of Antioxidant Activity as Measured by DPPH (mg TE/100 g DW), Phenol Content (mg GAE/100 g DW), Level of Chlorogenic Acid (μg Chlorogenic Acid/g DW), Flavonol Level (μg Rutin/g DW) and Phloretin Glycoside Level (μg Phloridzin/g DW) for Pressurized Liquid Extracts of Apple Pomace in a Temperature Range of 75 to 125 °C

	predicted value				
constant coefficient	DPPH	FCR	chlorogenic acid	flavonols	phloretin glycoside
intercept β_0	972	1458	544	1223	804
concn β_1	210	151	29		66
temp β_2		83	55		62
concn × temp β_{12}	156	−504	−109	−305	−175
concn × concn β_{11}	−301	−111	−34		
temp × temp β_{22}	−260	−77	−121	−358	−249
R^2	0.96	1.00	0.99	0.91	0.98
residual SD	68	25	15	108	39
residual SD as % mean	9.29	1.95	3.21	10.30	5.63
P value of lack of fit test	0.14	0.42	0.67	0.15	0.89

(**Figures 1a** and **1c**). The large increase in antioxidant activity by using a temperature higher than 150 °C was therefore probably mainly due to the production of melanoidins, since they are known for their brown color and their antioxidant activity (*34*).

In addition to melanoidins, hydroxymethylfurfural was formed when temperatures over 150 °C were applied. For example in sample 7 (50% ethanol; 153 °C) 0.25 mg HMF/g apple pomace was detected, while in sample 9 (25% ethanol; 193 °C) 10.71 mg HMF/g apple pomace was detected. In some food products, such as fruit juice, the level of HMF is monitored as an index of nonenzymatic browning and regarded as an indicator of quality loss (*35*). This would limit the application of the extract in certain foods as a possible food ingredient. In addition, concerns over the carcinogenicity of HMF have been reported. For instance, HMF induced precursors of colon cancer in rats (*36*), but did not generate toxicity in various *in vitro* assays. Nonetheless it would be logical to avoid the generation of HMF in a food ingredient, which is why a lower temperature range of 75 to 125 °C was also used to design a separate RSM experiment and optimize PLE.

Low Temperature Range 75 to 125 °C. Response values for each set of variable combinations were obtained (**Table 2**) and fitted to the second order polynomial model and the linear model. **Table 4** describes the effects of tested ethanolic extraction conditions on the levels of parameters of interest. Two outliers had to be removed from the response values of antioxidant activity as measured by DPPH and phenol content by FCR. One outlier had to be removed from the responses for the level of chlorogenic acid, the total levels of flavonols and phloretin glycoside in order to obtain valid models.

Statistical analyses by ANOVA determined what model fitted best, and this model was used subsequently. For all response values (antioxidant activity by DPPH, phenol content by FCR, chlorogenic acid level, level of the sum of flavonols and level of phloretin glucosides) the second order polynomial model was the best fit (**Table 4**). All models for PLE with a mild temperature range were found to be significant (p-value < 0.05), and all five models were adequate models, since the coefficient of determination (R^2) of these responses was greater than 0.91 (**Table 4**). **Table 4** shows that all models, except for the summed flavonol response, were affected by ethanol concentration (β_1). All models were affected by the temperature by ethanol concentration factor (β_{12}).

The model response in the lower temperature range was different from that of the higher range, i.e., an optimum activity

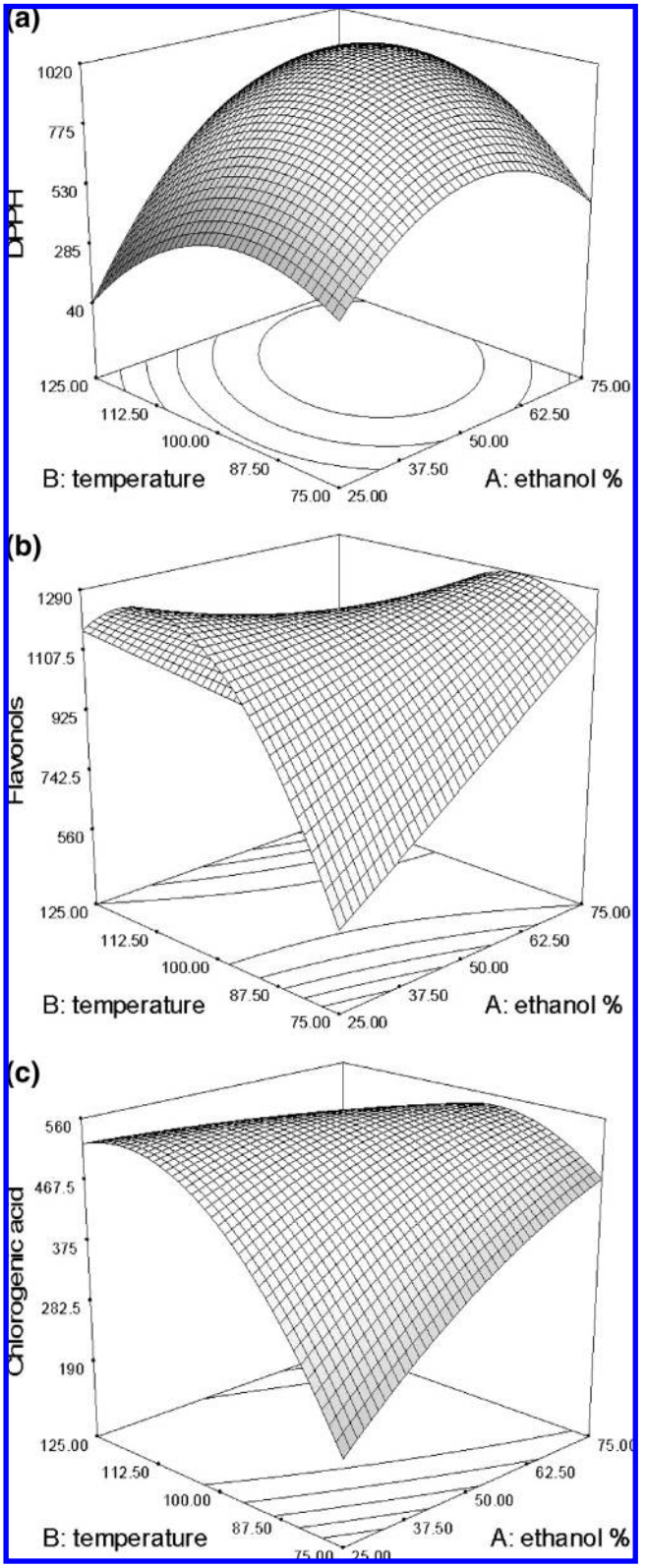

Figure 2. Response surface plot of PLE between 75 and 125 °C showing the effects of temperature and ethanol concentration on (**a**) antioxidant activity as measured by DPPH (mg TE/100 g DW apple pomace), (**b**) the level of flavonols (μg rutin/g DW apple pomace), and (**c**) the level of chlorogenic acid (μg chlorogenic acid/g DW apple pomace).

was found at 102 °C and 60% ethanol. At temperatures above 102 °C a decrease in the antioxidant activity of the extracts was observed (**Figure 2a**). This was expected since antioxidants measured such as phloretin glycosides and flavonols were also

Table 5. Optimal Conditions for Extracting Compounds with Antioxidant Activity and the Resulting Mean Response Values and Standard Deviations of Triplicate Extracts[a]

	response 1, DPPH		response 2, FCR		response 3, chlorogenic acid		response 4, FLA		response 5, PHLOR	
	pred	actual	pred	actual	pred	actual	pred	actual	pred	actual
60% ethanol, 102 °C	1011	1091 ± 82	1487	1442 ± 58	550	534 ± 21	1205	1372 ± 63	826	813 ± 18

[a] X_1 = ethanol concentration, X_2 = temperature (°C); X_3 = time (min); DPPH = antioxidant activity (mg TE/100 g DW); FCR = phenol level (mg GAE/100 g DW); CHA = level of chlorogenic acid (μg chlorogenic acid/g DW); FLA = sum of level of flavonols (μg rutin/g DW); PHLOR = level of phloretin glycoside (μg phloridzin/g DW).

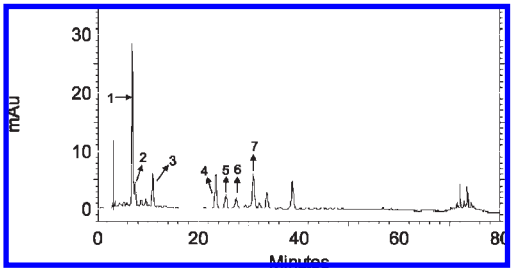

Figure 3. HPLC chromatogram at 320 nm of optimal pressurized extract of apple pomace at 102 °C and 60% ethanol. The peaks were identified as (1) chlorogenic acid, (2) caffeic acid, (3) *p*-coumaric acid, (4) quercetin glycoside, (5) rutin, (6) quercetin glycoside, (7) quercetin glycoside, and (8) phloretin glycoside.

degraded at higher temperatures (**Figure 2b**). Chlorogenic acid (**Figure 2c**) seemed to be degraded at temperatures higher than 112 °C (depending on the ethanol concentration). On the other hand an increase of chlorogenic acid levels was detected at temperatures higher than 160 °C (**Figure 1b**). It is known that melanoidins can noncovalently bind chlorogenic acid (*37*); therefore chlorogenic acid may be released again at these higher temperatures.

Since the nature of the response model of the antioxidant activity as measured by DPPH differed to the model responses for FCR, chlorogenic acid, flavonols and phloretin glycoside (**Table 4** and **Figures 2a** to **2c**), in addition to (poly)phenols there must be other compounds playing an important role in antioxidant activity.

Optimization Low Temperature Range. Since the preparation of extracts with high antioxidant activity would be one of the main purposes of the intended food ingredient, we developed optimal conditions for antioxidant activity as measured by DPPH. Optimization of antioxidant activity showed an optimum at 60% ethanol and 102 °C. The predicted and actual results are shown in **Table 5**. **Table 5** shows that RSM is a good method of optimizing extraction by PLE, since the optimal values were quite similar to what was predicted by the models. **Figure 3** shows a chromatogram of polyphenols present in an optimal extract obtained at 320 nm by HPLC. The figure shows a main peak of chlorogenic acid, which also has its maximum wavelength around 320 nm.

When compared to the optimal results obtained by traditional solid−liquid extraction of the same apple pomace with ethanol/water combinations, PLE resulted in better yields for antioxidants (*31*). The antioxidant activity of the optimal extracts was 2.4 times higher than the optimal extracts obtained by solid liquid extraction. These extracts showed also a higher yield of some of the polyphenol groups: the phenol content as measured by FCR was 1.3 higher, the summed level of flavonols 1.3 times higher and the level of phloretin glucoside 1.22 times higher. The level of chlorogenic acid was 0.95 times lower. These conditions were optimized for maximum antioxidant activity and polyphenol yields would increase if optima from RSM models for individual polyphenol groups were used.

In addition to higher extraction yields, a smaller amount of solvents and time is required in PLE versus conventional solid liquid extractions. The disadvantage is that a capital investment has to be made and that to our knowledge no large scale PLE extractors are available commercially at the moment.

In summary, PLE is a green extraction technique that can be used to enhance the antioxidant activity or polyphenol levels of a potential food ingredient. A temperature of 200 °C would be advantageous for antioxidant activity, but compounds like HMF will be formed. A lower temperature range between 75 and 125 °C is therefore recommended. A maximum of antioxidant activity was determined at 60% ethanol and 102 °C. By using PLE the antioxidant activity was increased 2.4 times in comparison to traditional solid−liquid extraction.

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