Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

Extraction of Total Phenolics of Sour Cherry Pomace by High Pressure Solvent and Subcritical Fluid and Determination of the Antioxidant Activities of the Extracts

İncinur Hasbay Adil[a]; M. Esra Yener[a]; Alev Bayındırlı[a]
[a] Department of Food Engineering, Middle East Technical University, Ankara, Turkey

To cite this Article Adil, İncinur Hasbay , Yener, M. Esra and Bayındırlı, Alev(2008) 'Extraction of Total Phenolics of Sour Cherry Pomace by High Pressure Solvent and Subcritical Fluid and Determination of the Antioxidant Activities of the Extracts', Separation Science and Technology, 43: 5, 1091 — 1110
To link to this Article: DOI: 10.1080/01496390801888243
URL: http://dx.doi.org/10.1080/01496390801888243

Separation Science and Technology, 43: 1091–1110, 2008

ISSN 0149-6395 print/1520-5754 online
DOI: 10.1080/01496390801888243

Extraction of Total Phenolics of Sour Cherry Pomace by High Pressure Solvent and Subcritical Fluid and Determination of the Antioxidant Activities of the Extracts

İncinur Hasbay Adil, M. Esra Yener, and Alev Bayındırlı

Department of Food Engineering, Middle East Technical University, Ankara, Turkey

Abstract: High pressure liquid extraction (HPE) and subcritical fluid (CO_2 + ethanol) extraction (SCE) were used for the extraction of total phenolic compounds (TPC) from sour cherry pomace. Antiradical efficiency (AE) of the extracts was also determined. Ethanol was the solvent for HPE and co-solvent for SCE. Combinations of pressure (50, 125, 200 MPa), temperature (20, 40, 60°C), solid/solvent ratio (0.05, 0.15, 0.25 g/ml) and extraction time (10, 25, 40 min) were variables for HPE according to the Box-Behnken experimental design. The variables used for SCE were pressure (20, 40, 60 MPa), temperature (40, 50, 60°C), ethanol concentration (14, 17, 20 wt%) and extraction time (10, 25, 40 min). For HPE, TPC, and AE at the optimum conditions (176–193 MPa, 60°C, 0.06–0.07 g solid/ml solvent, 25 min) were found as 3.80 mg gae/g sample and 22 mg $DPPH^{\bullet}$/g sample, respectively. TPC and AE at the optimum conditions (54.8–59 MPa, 50.6–54.4°C, 20 wt% ethanol, 40 min) for SCE were determined as 0.60 mg gae/g sample and 2.30 mg $DPPH^{\bullet}$/g sample for sour cherry pomace, respectively.

Keywords: High pressure extraction, subcritical fluid extraction, sour cherry, antioxidant activity, phenolic

Received 23 August 2007, Accepted 5 December 2007

Address correspondence to Alev Bayındırlı, Department of Food Engineering, Middle East Technical University, 06531 Ankara, Turkey. Tel.: +90-312 210 5629;. Fax: +90-312 210 1270; E-mail: alba@metu.edu.tr

INTRODUCTION

Fruits and vegetables are the best carriers of vitamins and polyphenols. Nowadays, their industrial wastes are attractive sources of natural antioxidants (1–4). Until recent years, extraction of antioxidants have been done by simple conventional solvent extraction (usually Soxhlet extraction) using several solvents like ether, ethanol, ethyl acetate, acetone, and water (2, 5–10).

Pressurized liquid extraction is a method that uses organic solvents at pressures up to 15 MPa and temperatures above the boiling point of solvent for short periods of time with reduced solvent consumption (11). High pressure increases contact between the extracting fluid and the sample, allows disruption of the solute-matrix interaction, and provides a possibility to remove air blocks in the material with an increase in the diffusion rates (12, 13). In the study of Palma et al. (14), the stability of phenolic compounds (p-coumaric acid, vanillin, veratric acid, protocatechuic aldehyde, gentisic acid, caffeic acid, syringic aldehyde, catechin, and epicatechin) in the extraction with methanol at 10 MPa and different temperatures (40–150°C) was tested by using a model system. After three 10 min cycles, all the assayed phenolic compounds were stable under the extraction conditions with the exception of catechin and epicatechin. Elevated temperatures are reported to improve the efficiency of extraction due to enhanced diffusion rate and solubility. However, elevated extraction temperatures may simultaneously increase the rate of degradation. Conventional extraction of anthocyanins is typically conducted at temperatures ranging from 20 to 50°C. Besides acid concentration and temperature, additional factors such as light, oxygen, metals, sugars, and their degradation products have been shown to affect the stability and antioxidant capacity of anthocyanins (15).

Supercritical and/or subcritical extraction is an alternative extraction method for the food industry due to the advantages such as non-toxicity and easy removal of solvent. Moreover, the absence of air during extraction can reduce the risk of degradation reactions that is possible during the extraction of phenolic compounds. CO_2 is generally the most desirable solvent for extraction. Addition of organic co-solvents like ethanol and methanol increases the polarity of CO_2 and the yield of extraction of polar phenolic compounds. When a co-solvent is added to CO_2, the critical temperature of the resulting mixture is increased while the critical pressure is decreased. If the elevated temperatures are not preferred, the extraction is performed under the critical temperature of the mixture that is called subcritical fluid extraction. Supercritical and/or subcritical extraction with ethanol and methanol as co-solvent have been used for extraction of phenolic compounds from grape seeds (16, 17). The solubility of hydroxycinnamic acids such as p-coumaric acid, caffeic acid, and ferrulic acid (18), quercetin (19), catechin (20), epicathecin (21), and resveratrol (22) in supercritical CO_2 are available. Ethanol (5–30%) is the co-solvent used to increase the

solubility of polyphenols in CO_2 except the phenolic acids. Therefore, the extraction of phenolic compounds other than phenolic acids needs to be subcritical between 40–60°C.

Total phenolics are concentrated in the skin of sour cherries (23). They contain significant level of anthocyanins. The polyphenolic compounds: 5,7,4′-trihydroxyflavanone, 5,7,4′-trihydroxyisoflavone, chlorogenic acid, 5,7,3′,4′-tetrahydroxy-flavonol-3-rhamnoside, 5,7,4′-trihydroxyflavonol 3-rutinoside, 5,7,4′-trihydroxy-3′methoxyflavonol-3-rutinoside, 5,7,4′-trihydroxyisoflavone-7-glucoside, and 6,7-dimethoxy-5,8,4′-trihydroxyflavone were determined in sour cherries by NMR experiments. The antioxidant assays revealed that 7-dimethoxy-5,8,4′-trihydroxyflavone is the most active one, followed by quercetin 3-rhamnoside, genistein, chlorogenic acid, naringenin, and genistin (24). Wang et al. (25) found three novel compounds, 2-hydroxy-3-(o-hydroxyphenyl) propanoic acid, 1-(3′,4′-dihydroxycinnamoyl)-cyclopenta-2,5–diol and 1-(3′,4′-dihydroxycinnamoyl)-cyclopenta-2,3-diol which have strong antioxidant activity. The average total antioxidant concentration in sour cherries obtained from different sources such as geographical location or manufactures is 5.53 mmol/100 g fresh weight according to FRAP (reduction of Fe^{+3} to Fe^{+2}) assay (26). Blando et al. (27) reported that a relatively high antioxidant capacity for the fruit extracts, measured as ORAC assay, ranged from 1145 to 1916 μmol Trolox equivalent/100 g fresh weight.

The aim of this work is to extract phenolic compounds from the sour cherry pomace by high pressure extraction (HPE) at temperatures lower than the boiling point but pressures higher then used in pressurized liquid extraction, subcritical fluid extraction (SCE) and solvent extraction (SE) by considering the total amount of the extracted phenolic compounds and the antioxidant activity and to optimize the extraction.

MATERIALS AND METHODS

The sour cherry pomace obtained from the fruit juice production pilot plant of Ankara University, Food Engineering Department. After washing, removal of stems, crashing, heating (85–90°C) and pressing, the pomace in polyethylene plastic bags was stored at −35°C and freeze-dried at −5°C (Model FD8, Heto Lab. Equipment, Allerød, Denmark). The moisture content of the dried pomace was $14 \pm 0.75\%$ ($n = 5$). The dried samples were ground using kitchen-type grinder (Moulinex, France). The average particle size of samples was 0.638 mm by sieve analysis (Endecotts Ltd, London, England).

High Pressure Extraction (HPE)

HPE was performed in a designed and constructed lab-scale unit (capacity: 30 cm^3). The rate of pressure increase and pressure release was approximately

5–10 s for the designed system. Water was used as pressure transmitting medium. The equipment consists of a pressure chamber of cylindrical design, two end closures, a means for restraining the end closures, a pressure pump, and a hydraulic unit to generate high pressure for system compression and also a temperature control device.

Fresh ethanol (99.8%, Riedel, Inc., Steinheim, Germany) was used as solvent during batch extraction. For pressurization, the 3 ml vials were completely filled with solid and solvent at the solid/solvent ratio required according to the experimental design by avoiding air bubbles as much as possible. The experimental design was three level Box-Behnken design (28) with four independent variables that were pressure (50, 125, and 200 MPa), temperature (20, 40, and 60°C), solid/solvent ratio (0.05, 0.15, and 0.25 g/ml) and extraction time (10, 25, and 40 min). The experimental design points were given in Table 1.

Subcritical Fluid (CO_2 + Ethanol) Extraction (SCE)

Extractions were performed by a Supercritical Fluid Extraction System (SFX System 5100, ISCO Inc., Lincoln, NE, USA), which consists of an extractor (SFX 3560) and two syringe pumps (Model 100DX) that enables co-solvent addition. 1 gram of sample was placed into 10 ml sample cartridge. Ethanol and CO_2 (99.9%; Bos, İstanbul, Turkey) mixture was used as a solvent for the extractions. The variables were pressure (20, 40, and 60 MPa), temperature (40, 50, and 60°C), the amount of ethanol in CO_2 (14, 17, and 20 wt%) and extraction time (10, 25, and 40 min). The Box-Behnken design was given in Table 2. The solvent flow (2 g/min) was downward. The restrictor temperature was 80°C. All the extracts were collected in ethanol (3 ml).

Solvent Extraction (SE)

Ethanol and methanol (99.8%, Riedel, Inc., Steinheim, Germany) was used for SE for comparison. Different mixtures with solid to solvent ratios 0.05, 0.1, 0.2, and 0.3 g/ml were prepared by adding 4 ml solvent on 0.2, 0.4, 0.8, and 1.2 g sample, respectively. The most general methodology for extraction of phenolic compounds for analysis involves the use of aqueous methanol for 16–24 h at room temperature (16, 29). The mixtures were kept at room temperature in dark for 24 h.

Determination of Total Phenolic Content

Folin-Ciocalteu method was used for the determination of TPC (30). The absorbance measurements were done at 740 nm (Pharmacia LKB Novaspec II model

Table 1. Coded and uncoded levels of Box-Benken design and TPC and AE of the extracts obtained by HPE[a]

Exp. no[b]	X_1 (MPa)	X_2 (°C)	X_3 (g/ml)	X_4 (min)	TPC (mg gae/g sample)	AE (mg $DPPH^{\bullet}$/ g sample)
1	+1 (200)	+1 (60)	0 (0.15)	0 (25)	3.26	17.31
2	+1 (200)	−1 (20)	0 (0.15)	0 (25)	2.28	12.58
3	−1 (50)	+1 (60)	0 (0.15)	0 (25)	2.66	14.67
4	−1 (50)	−1 (20)	0 (0.15)	0 (25)	2.22	13.77
5	0 (125)	0 (40)	+1 (0.25)	+1 (40)	3.01	10.10
6	0 (125)	0 (40)	+1 (0.25)	−1 (10)	2.37	9.36
7	0 (125)	0 (40)	−1 (0.05)	+1 (40)	2.82	19.23
8	0 (125)	0 (40)	−1 (0.05)	−1 (10)	2.62	15.87
9	0 (125)	0 (40)	0 (0.15)	0 (25)	2.70	14.67
10	+1 (200)	0 (40)	0 (0.15)	+1 (40)	2.90	16.25
11	+1 (200)	0 (40)	0 (0.15)	−1 (10)	2.73	13.81
12	−1 (50)	0 (40)	0 (0.15)	+1 (40)	2.44	14.80
13	−1 (50)	0 (40)	0 (0.15)	−1 (10)	2.36	11.91
14	0 (125)	+1 (60)	+1 (0.25)	0 (25)	2.71	12.13
15	0 (125)	+1 (60)	−1 (0.05)	0 (25)	3.73	21.03
16	0 (125)	−1 (20)	+1 (0.25)	0 (25)	2.11	9.80
17	0 (125)	−1 (20)	−1 (0.05)	0 (25)	1.84	15.82
18	0 (125)	0 (40)	0 (0.15)	0 (25)	2.79	13.70
19	+1 (200)	0 (40)	+1 (0.25)	0 (25)	2.31	11.44
20	+1 (200)	0 (40)	−1 (0.05)	0 (25)	2.99	20.52
21	−1 (50)	0 (40)	+1 (0.25)	0 (25)	2.25	11.83
22	−1 (50)	0 (40)	−1 (0.05)	0 (25)	2.38	19.08
23	0 (125)	+1 (60)	0 (0.15)	+1 (40)	3.32	16.43
24	0 (125)	+1 (60)	0 (0.15)	−1 (10)	3.38	15.33
25	0 (125)	−1 (20)	0 (0.15)	+1 (40)	2.55	15.03
26	0 (125)	−1 (20)	0 (0.15)	−1 (10)	1.79	9.84
27	0 (125)	0 (40)	0 (0.15)	0 (25)	2.74	14.63

[a]X_1, X_2, X_3, and X_4 represent coded levels of pressure, temperature, solid/solvent ratio and extraction time, respectively. Variable uncoded levels are given in parenthesis.
[b]Experiments were performed in random order.

UV-spectrophotometer, UK). TPC was expressed as gallic acid equivalent using the standard curve prepared at different concentrations of gallic acid (98%, Acrös Organics, Belgium) and given as mg gallic acid equivalent (gae)/g sample. The g sample refers to the grams of the freeze dried pomace.

Determination of Antioxidant Activity

Antioxidant activity of the extracts was determined by detecting the scavenging of $DPPH^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl, (Sigma, Germany)) radical

Table 2. Coded and uncoded levels of Box-Benken design and TPC and AE of the extracts obtained by SCE[a]

Exp. no[b]	X_1 (MPa)	X_2 (°C)	X_3 (wt%)	X_4 (min)	TPC (mg gae/g sample)	AE (mg $DPPH^{\bullet}$/ g sample)
1	+1 (60)	+1 (60)	0 (17)	0 (25)	0.300	1.038
2	+1 (60)	−1 (40)	0 (17)	0 (25)	0.324	1.149
3	−1 (20)	+1 (60)	0 (17)	0 (25)	0.264	0.932
4	−1 (20)	−1 (40)	0 (17)	0 (25)	0.179	0.607
5	0 (40)	0 (50)	+1 (20)	+1 (40)	0.542	1.863
6	0 (40)	0 (50)	+1 (20)	−1 (10)	0.298	1.010
7	0 (40)	0 (50)	−1 (14)	+1 (40)	0.258	0.923
8	0 (40)	0 (50)	−1 (14)	−1 (10)	0.188	0.439
9	0 (40)	0 (50)	0 (17)	0 (25)	0.353	1.103
10	+1 (60)	0 (50)	0 (17)	+1 (40)	0.396	1.613
11	+1 (60)	0 (50)	0 (17)	−1 (10)	0.139	0.502
12	−1 (20)	0 (50)	0 (17)	+1 (40)	0.282	1.056
13	−1 (20)	0 (50)	0 (17)	−1 (10)	0.099	0.363
14	0 (40)	+1 (60)	+1 (20)	0 (25)	0.419	1.604
15	0 (40)	+1 (60)	−1 (14)	0 (25)	0.256	0.909
16	0 (40)	−1 (40)	+1 (20)	0 (25)	0.495	1.579
17	0 (40)	−1 (40)	−1 (14)	0 (25)	0.170	0.551
18	0 (40)	0 (50)	0 (17)	0 (25)	0.349	1.220
19	+1 (60)	0 (50)	+1 (20)	0 (25)	0.503	2.000
20	+1 (60)	0 (50)	−1 (14)	0 (25)	0.228	0.860
21	−1 (20)	0 (50)	+1 (20)	0 (25)	0.283	1.071
22	−1 (50)	0 (50)	−1 (14)	0 (25)	0.126	0.405
23	0 (40)	+1 (60)	0 (17)	+1 (40)	0.446	1.724
24	0 (40)	+1 (60)	0 (17)	−1 (10)	0.150	0.541
25	0 (40)	−1 (40)	0 (17)	+1 (40)	0.261	0.826
26	0 (40)	−1 (40)	0 (17)	−1 (10)	0.157	0.486
27	0 (40)	0 (50)	0 (17)	0 (25)	0.353	1.056

[a]X_1, X_2, X_3, and X_4 represent coded levels of pressure, temperature, ethanol amount in CO_2 and extraction time, respectively. Variable uncoded levels are given in parenthesis.

[b]Experiments were performed in random order.

(31, 32). Different amounts of extracts (0–0.2 ml) were placed in tubes followed by evaporation of ethanol in the extracts in dark at room temperature. 0.1 ml of methanol was placed in the tubes. The tubes were mixed properly to allow the antioxidants dissolve in methanol and 3.9 ml of 0.025 mg/ml $DPPH^{\bullet}$ solution was added. After holding the tubes for one hour in the dark at room temperature, the absorbance values were measured at 515 nm (Pharmacia LKB Novaspec II model UV-spectrophotometer, UK) and converted to $DPPH^{\bullet}$ concentration using the standard

curve prepared for each set of experiment. The percentage of remaining $DPPH^{\bullet}$ was calculated as

$$\%DPPH^{\bullet}_{rem} = ([DPPH^{\bullet}]_t/[DPPH^{\bullet}]_{t=0}) \times 100 \quad (1)$$

The percentage of the remaining $DPPH^{\bullet}$ against the sample concentration was then plotted to determine EC_{50} (efficient concentration of the sample to decrease the initial $DPPH^{\bullet}$ concentration by 50%). The antioxidant activity was expressed in terms of antiradical efficiency (AE) which is defined as $AE = 1/EC_{50}$ (33–36) and given as AE/g sample. The g sample refers to the grams of the freeze dried pomace.

Data Analysis

Second-order polynomial equations were used to express the TPC (Y_1, mg gallic acid equivalent/g sample) and AE (Y_2, mg $DPPH^{\bullet}$/g sample) of extracts as follows:

$$\begin{aligned} Y = {} & a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 \\ & + a_{44}X_4^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{23}X_2X_3 \\ & + a_{24}X_2X_4 + a_{34}X_3X_4 \end{aligned}$$

where X_1, X_2, X_3, and X_4 represent the codes of pressure, temperature, solid/solvent ratio and time for HPE or the codes of pressure, temperature, ethanol amount in CO_2, and time for SCE, respectively. The coefficients of the response functions and also the statistical significance of results were determined by using the package program MINITAB 13.20. Contour plots were created using Surfer 6.01. Contour plots were superimposed to estimate the optimum extraction conditions.

RESULTS AND DISCUSSION

High Pressure Extraction (HPE)

Ethanol was selected as the solvent due to its low toxicity considering the possible future applications of the extracted phenolic compounds in food products. Tables 1, 2, and 3 show the experimental data and the regression coefficients obtained by fitting experimental data to the second order response models for TPC and AE of the extracts obtained by HPE. Figures 1 and 2 represent example response surfaces obtained for HPE. As a result of the t-tests for $p < 0.05$, all independent variables were found to have a significant effect on TPC and AE of the extracts. Alonso-Salcez et al. (11) reported that the effect of pressure in the range of 6.9–10.3 MPa

Table 3. Second order response model constants and regression analysis for TPC and AE of the extracts obtained by HPE

	HPE		SCE	
Model constants[a]	TPC (mg gae/g sample)	AE (mg $DPPH^{\bullet}$/g sample)	TPC (mg gae/g sample)	AE (mg $DPPH^{\bullet}$/g sample)
a_0	2.743**	14.333**	0.3517**	1.1263**
a_1	0.180**	0.488*	0.0547**	0.2273**
a_2	0.523**	1.672**	0.0207*	0.1292**
a_3	−0.135**	−3.908**	0.1095**	0.4200**
a_4	0.149**	1.310**	0.0962**	0.3887**
a_{11}	−0.150	0.582	−0.0673**	−0.1158
a_{22}	−0.017	0.053	−0.0300	−0.0720
a_{33}	−0.105	0.362	0.0131	0.0865
a_{44}	0.038	−0.669*	−0.0556**	−0.1470*
a_{12}	0.135	0.958*	−0.0272	−0.1090
a_{13}	−0.138	−0.458	0.0295	0.1185
a_{14}	0.023	−0.113	0.0185	0.1045
a_{23}	−0.323**	−0.720*	−0.0405*	−0.0832
a_{24}	−0.205*	−1.023**	0.0480**	0.2108**
a_{34}	0.110	−0.655	0.0435*	0.0922
R^2	0.941	0.979	0.965	0.961
F	13.58	40.92	23.61	21.36
Sig F	0.000	0.000	0.000	0.000
Std. Error	0.165	0.674	0.03349	0.1348

[a] $y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{44}X_4^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{34}X_3X_4$.

*Significant at $p \leq 0.05$.

**Significant at $p \leq 0.01$.

was insignificant on the extraction of phenolic compounds from apple peel and pulp at 40°C for 5 min with a total volume of 25 ml of methanol for 1 g sample. In our study, the effect of pressure (50–200 MPa) on TPC and AE of the extracts was positive for all samples. Elevated pressures facilitate solvent penetration through the interior of the sample matrix, therefore increases mass transfer rate (13).

Generally, high temperature increases the solubility and diffusion coefficients of the compounds to be extracted (12) and decreases the viscosity of the solvent, which facilitates its penetration through the solid matrix (37). However, the temperature range to be studied should be selected carefully so that the heat sensitive phenolic compounds should not be inactivated. Therefore, high pressures than used in pressurized liquid extraction, were applied during this study. The temperature range was selected as 20–60°C to prevent the loss of phenolic compounds at elevated temperatures. Ju and

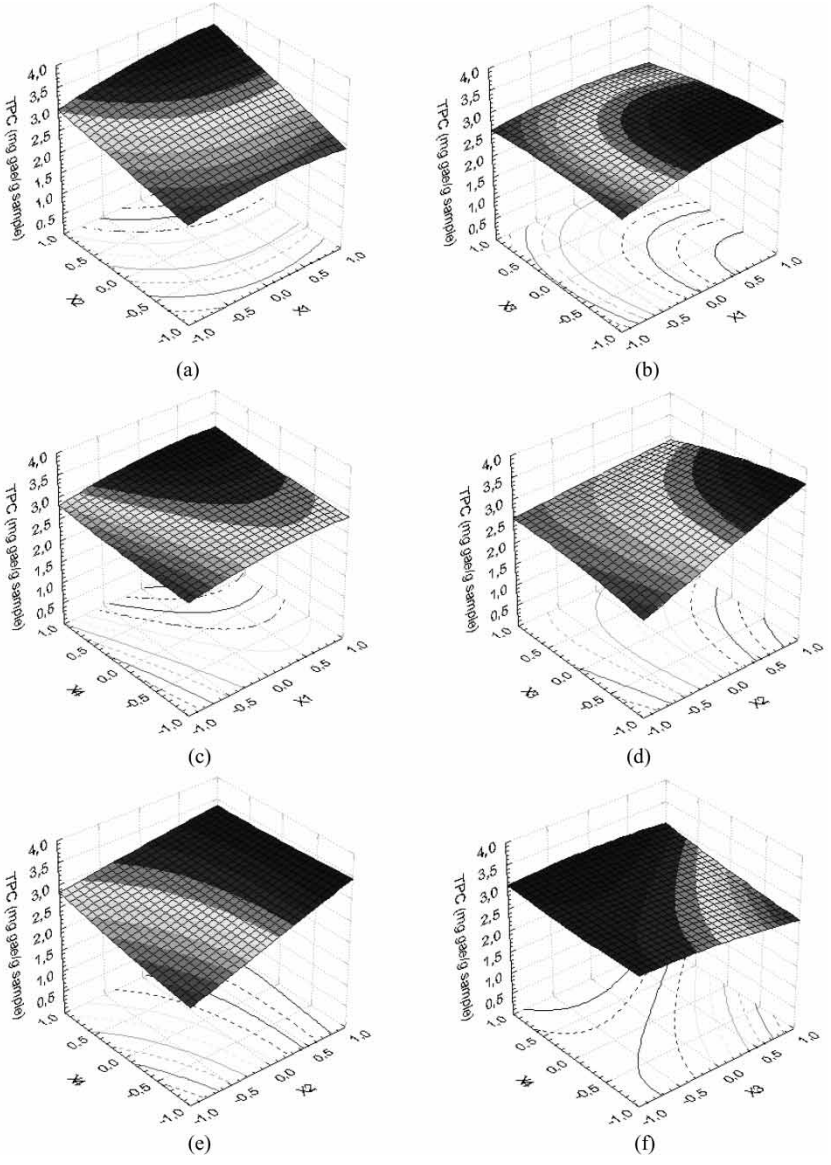

Figure 1. Response surface examples of TPC (mg gae/g sample) of the extracts from sour cherry pomace obtained by HPE (a) $X_3 = X_4 = 0$, (b) $X_2 = X_4 = 0$, (c) $X_2 = X_3 = 0$, (d) $X_1 = X_4 = 0$, (e) $X_1 = X_3 = 0$, (f) $X_1 = X_2 = 0$.

Howard (15) used pressurized liquid extraction to extract anthocyanins from the freeze-dried skin of a highly pigmented red wine grape with different solvents. Optimum temperatures for the extraction of total anthocyanins were determined as 80–100°C for acidified water and 60°C for acidified

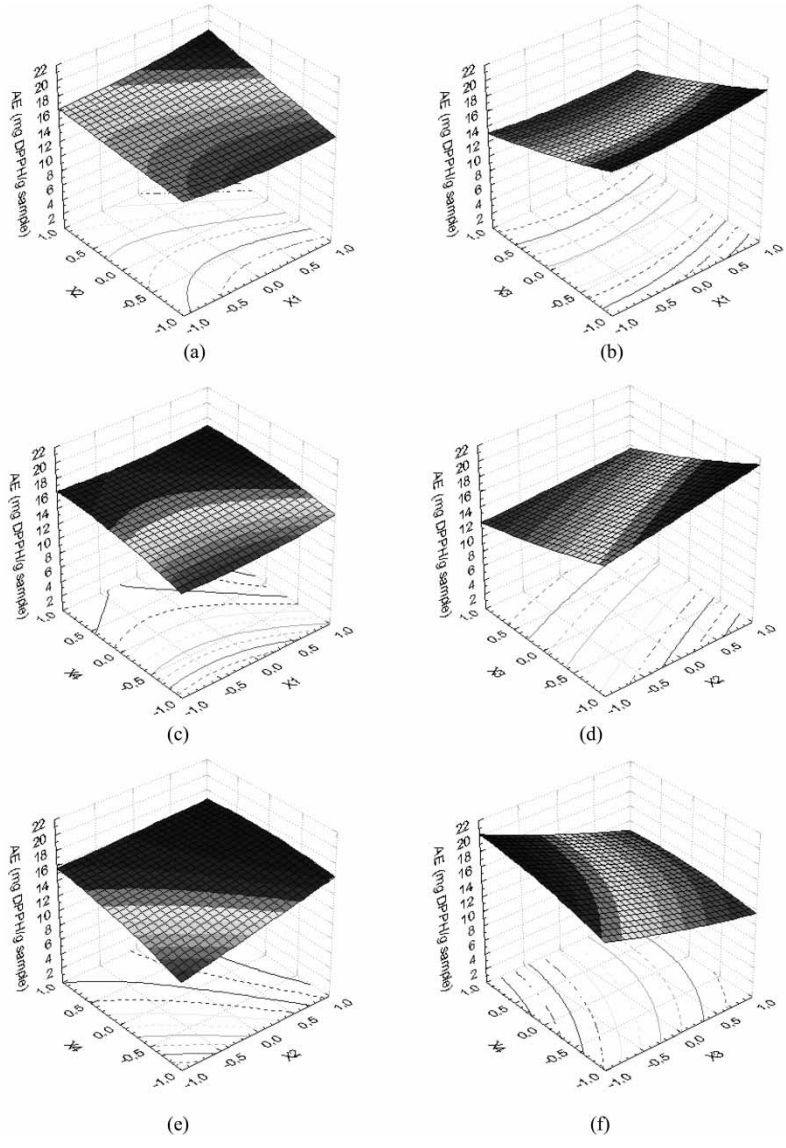

Figure 2. Response surface examples of AE (mg $DPPH^{\bullet}$/g sample) of the extracts from sour cherry pomace obtained by HPE (a) $X_3 = X_4 = 0$, (b) $X_2 = X_4 = 0$, (c) $X_2 = X_3 = 0$, (d) $X_1 = X_4 = 0$, (e) $X_1 = X_3 = 0$, (f) $X_1 = X_2 = 0$.

60% methanol. They showed that total anthocyanins were degraded at temperatures greater than 100°C. On the other hand, Cacace and Mazza (38), working on extraction on anthocyanins from milled berries in an agitated vessel, found that there was a sharp decrease in the amount of

anthocyanins extracted at temperatures higher than 45°C for about 125–275 min. In the temperature range studied (6–74°C), the maximum anthocyanin extraction was obtained at 30–35°C using ethanol as a solvent. Alonso-Salcez et al. (11), indicated the inactivation of phenolic compounds above 60°C during the pressurized extraction of phenolic compounds from apple peel and pulp with methanol.

Increasing solid/solvent ratio decreased the TPC and AE for all samples, which may be due to the low concentration gradient at high solid/solvent ratios. Cacace and Mazza (38), working on the mass transfer process during the extraction of anthocyanins from milled berries, examined the effect of solid/solvent ratio (0.0135–0.1667 g/ml) on the amount of anthocyanins extracted at 40°C with ethanol and found that the extraction yield was higher at low solid/solvent ratios. Concentration gradient, i.e., the driving force during mass transfer within the solid, was greater when a lower solid-solvent ratio was used. Similar effects were seen by Rostagno et al. (39), working on the extraction of isoflavones from soybeans by accelerated solvent extractor. They found that the extraction efficiency of some isoflavones constantly increased with the reduction of the amount of sample from 0.5 to 0.05 g in a total volume of 22 ml in the continuous extraction system. They also evaluated the stability of isoflavones from soybeans at elevated temperatures at 10 MPa for 3 × 5 min cycles and their results showed that 100°C is the maximum temperature for extraction of isoflavones.

Superimposition of contour plots (not shown here) were done to determine the optimum regions. The optimum extraction conditions were presented in Table 4. Kim et al. (40) extracted phenolic compounds from sweet and sour cherry by using homogenization and sonication with methanol and found that the amount of phenolic compounds in different sour cherry varieties range between 1.617–3.124 mg gae/g fresh weight. If the result is converted to dry weight basis using the moisture content of cherries as 81% (41), TPC for sour cherries is 8.51–16.44 mg gae/g dry weight according to the findings of Kim et al. (40). Those results are higher than our findings (3.8 mg gae/g sample) due to the sample difference (fresh fruit and pomace).

Subcritical Fluid (CO_2 + Ethanol) Extraction (SCE)

The ethanol concentration range and the levels of the design variables were decided by performing preliminary extractions at 60 MPa, 60°C, using 2 g/min solvent flow rate for 30 minutes with 0–20 wt% ethanol in CO_2. At 60°C by 0–10 wt% ethanol addition, supercritical CO_2 extraction was performed where TPC and AE of the extracts were low. TPC and AE of the extracts increased about four-fold at higher ethanol concentrations, where the extraction was subcritical. Therefore, it was decided that subcritical CO_2 was advantageous than supercritical CO_2 extraction. The extraction time was

Table 4. Optimum conditions for HPE and SCE

Factor	Coded level	Uncoded level
HPE		
Pressure	X_1 : 0.68–0.90	176–193 MPa
Temperature	X_2 : 1	60°C
Solid/solvent ratio	X_3 : −0.9– −0.78	0.06–0.07 g/ml
Time	X_4 : 0	25 min
Optimum responses		
TPC (mg gae/g sample) : 3.80		
AE (mg $DPPH^{\bullet}$/g sample) : 22.00		
AE/TPC (mg $DPPH^{\bullet}$/mg gae) : 5.79		
SCE		
Pressure	X_1 : 0.74–0.95	54.8–59MPa
Temperature	X_2 : 0.06–0.44	50.6–54.4°C
Ethanol concentration	X_3 : 1	20 wt%
Time (min)	X_4 : 1	40 min
Optimum responses		
TPC (mg gae/g sample) : 0.60		
AE (mg $DPPH^{\bullet}$/g sample) : 2.30		
AE/TPC (mg $DPPH^{\bullet}$/mg gae) : 3.83		

decided by performing extractions at 60 MPa, 60°C, using 2 g/min solvent flow rate for about 120 minutes. The maximum extraction time was selected to be 40 minutes to assure that TPC of the extracts increase significantly with extraction time at all extraction conditions.

Table 2 and 3 show the experimental data and the regression coefficients obtained by fitting experimental data to the second order response models for TPC and AE of the extracts obtained by SCE. t-tests for $p < 0.05$ show that all independent variables were significant for the extraction. Figures 3 and 4 represent example response surfaces for the TPC and AE of the extracts obtained from sour cherry pomace by SCE. The effect of pressure on TPC and AE of the extracts was positive. This is mainly due to the increase in the density of CO_2, i.e., increase in the solvating power with increasing pressure (12, 42). The effect of pressure was more significant at high ethanol concentration. The same result was obtained by Cháfer et al. (21) who work on the solubility of epicatechin in supercritical CO_2 + ethanol at 40°C and 8–14 MPa. As the concentration of ethanol increased from 5 to 25%, a sharper increase in solubility was observed with an increase in pressure. Murga et al. (17) determined the effect of pressure (10–50 MPa) on the solubility of some natural, low molecular weight phenolic compounds in supercritical CO_2 at 40–60°C and found that solubility increased with increasing pressure. Other researches on the solubility of catechin (20), epicatechin (21), quercetin (19) and resveratrol (22) in supercritical CO_2 + ethanol at 40°C and 8–14 MPa showed that pressure had a positive effect on solubility of the phenolic compounds of interest.

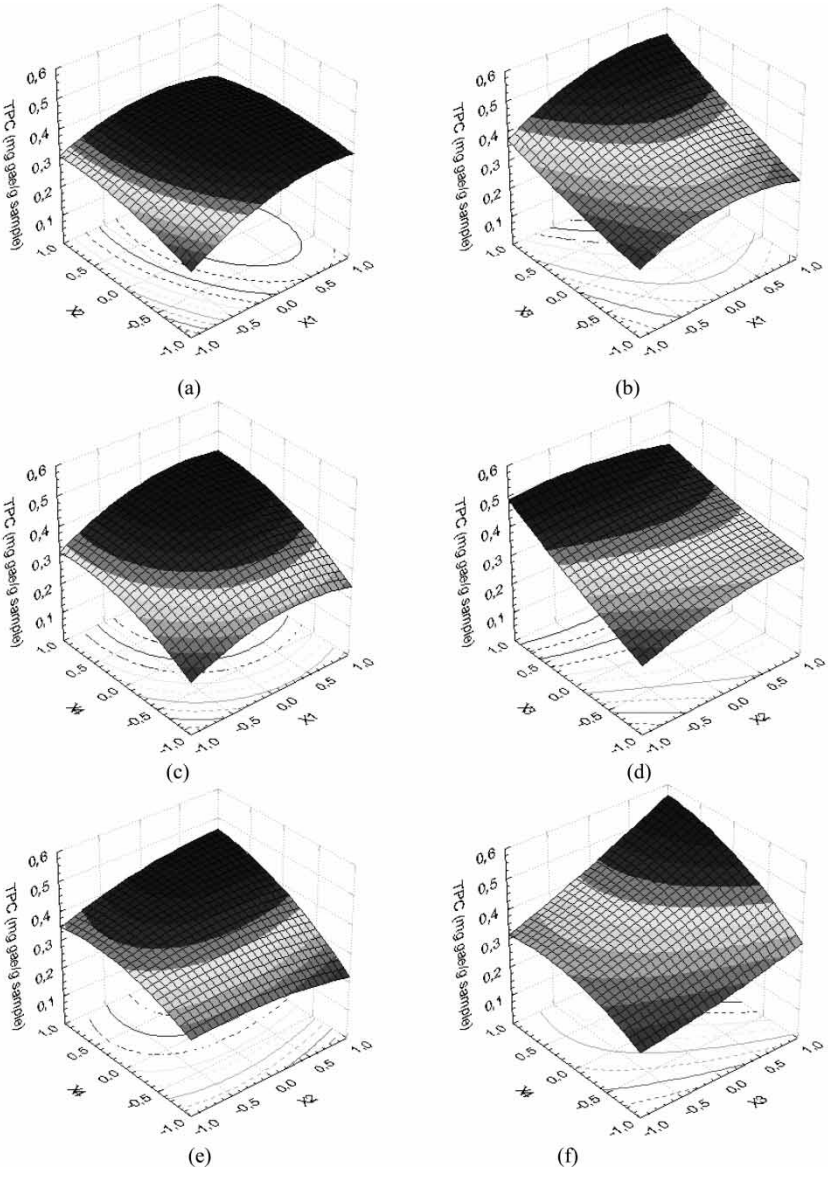

Figure 3. Response surface examples of TPC (mg gae/g sample) of the extracts from sour cherry pomace obtained by SCE (a) $X_3 = X_4 = 0$, (b) $X_2 = X_4 = 0$, (c) $X_2 = X_3 = 0$, (d) $X_1 = X_4 = 0$, (e) $X_1 = X_3 = 0$, (f) $X_1 = X_2 = 0$.

Temperature also had a positive effect on TPC and AE of the extracts. Generally, the effect of temperature is negative on the extraction of polyphenols at low pressures (10–15 MPa) up to a certain value, beyond which the effect of temperature becomes positive (17). The pressure value at which

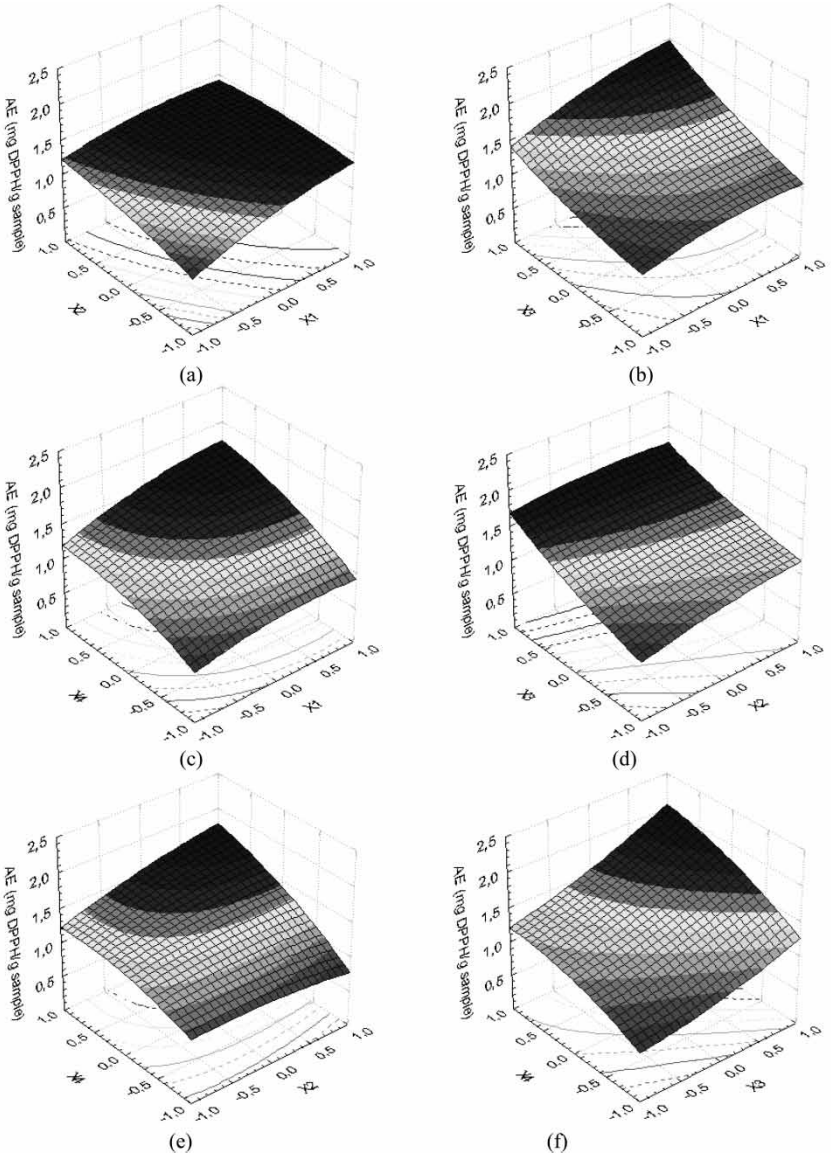

Figure 4. Response surface examples of AE (mg $DPPH^{\bullet}$/g sample))of the extracts from sour cherry pomace obtained by SCE (a) $X_3 = X_4 = 0$, (b) $X_2 = X_4 = 0$, (c) $X_2 = X_3 = 0$, (d) $X_1 = X_4 = 0$, (e) $X_1 = X_3 = 0$, (f) $X_1 = X_2 = 0$.

the effect of temperature on the solubility changes is called the cross-over pressure (43). This cross-over pressure arises from the solubility being controlled by a balance between the solvent density and the change in the solute vapour pressure with increase in temperature (44). This phenomenon

was not seen in this study because the extraction pressures were higher than the possible cross-over pressure. Also, Palma and Taylor (16) observed an increase in the recovery of phenolic compounds from grape seeds with near critical CO_2 with an increase in temperature from 35 to 55°C with 10% methanol and CO_2 density of 0.95 g/ml.

Ethanol concentration in CO_2 affected both responses positively as expected since the polarity of CO_2 increases as the ethanol concentration is increased, which results with more hydrogen bonding and dipole-dipole interactions, i.e., increased solubility of phenolic compounds. The effect of ethanol concentration on the solubility of phenolic compounds depend on their polarity. The solubility of catechin, epicatechin, quercetin, and resveratrol as a function of ethanol concentration was studied previously (19–22). All phenolic compounds showed different solubility behaviors with respect to ethanol concentration. For this study, the optimum extraction conditions were presented in Table 4 according to superimposed contour plots (not shown here).

Comparison of HPE, SCE, and SE

TPC and AE of the extracts obtained by HPE and SCE at optimum conditions were compared with those obtained by methanol and ethanol SE (Table 5). Different mixtures with solid/solvent ratios 0.05, 0.1, 0.2, and 0.3 g/ml were prepared which was in parallel with the solid/solvent ratios used in HPE (0.05–0.25 g/ml). As expected, methanol was a better solvent for the extraction of phenolic compounds which was in parallel with the findings in the literature (3, 21, 45). A positive significant ($p < 0.01$) correlation between TPC and AE of the extracts was found for SE with methanol and ethanol. The correlations (r values) are 0.99 and 0.89 for SE extraction with methanol and ethanol, respectively. Compared to SE, SCE was not so efficient in the extraction of phenolic compounds from sour cherry pomace

Table 5. TPC and AE of the extracts obtained by SE

Extraction method	Solid/solvent ratio (g/ml)	TPC (mg gae/g sample)	AE (mg $DPPH^{\bullet}$/ g sample)	AE/TPC (mg $DPPH^{\bullet}$/mg gae)
SE with methanol	0.05	3.52	25.6	7.27
	0.1	3.21	12.5	3.89
	0.2	3.01	4.60	1.53
	0.3	2.96	2.97	1.00
SE with ethanol	0.05	2.92	24.8	8.49
	0.1	2.62	12.1	4.62
	0.2	2.50	4.50	1.80
	0.3	2.06	2.87	1.39

in spite of the increased polarity of CO_2 with the addition of 20% (w/w) ethanol. This result may be due to the selectivity of SCE according to the polarity of phenolic compounds. There was a positive significant relationship ($p < 0.01$) between TPC and AE of the extracts with high correlation (0.97) for SCE.

HPE of phenolic compounds with ethanol at optimum conditions (solid/solvent ratio: 0.06–0.07 g/ml) yielded much higher TPC (3.80 mg gae/g sample) than that of SE with ethanol (2.92 and 2.62 mg gae/g sample for solid to solvent ratios of 0.05 and 0.1 g/ml, respectively) and they are close to those obtained by SE using methanol with a solid/solvent ratio of 0.05 g/ml. Using ethanol as a solvent at high pressure enhanced extraction. The AE value of the extract obtained by HPE at the optimum conditions was 22 mg $DPPH^{\bullet}$/g sample. The AE values were found as 24.8 and 12.1 mg $DPPH^{\bullet}$/g sample for SE with ethanol at a solid to solvent ratio of 0.05 and 0.1 g/ml, respectively. Although high correlation was obtained for SE, a positive significant ($p < 0.01$) correlation with a lower r value of 0.56 was obtained between TPC and AE of the extracts for HPE. This might be due to the synergistic effect among the free phenolic antioxidants or due to the contribution of non-phenolic antioxidative compounds such as ascorbic acid. However, Gil at al. (5) and Cevallos-Casals et al. (46) showed that phenolics in peaches were the only compounds that correlated with antioxidant capacity when compared with vitamin C and carotenoids. Ascorbic acid had only a minor contribution to the antioxidants in fruits with the exception of citrus fruits and strawberry and was destroyed during extraction under heat treatment (47). Ascorbic acid is also slightly soluble in ethanol, therefore the interference from ascorbic acid may be accepted as negligible. In addition, a statistically significant decrease in ascorbic acid content was reported in freeze dried marionberry and strawberry in the study of Asami et al. (48) related to extraction of phenolic compounds by acetone, water and acetic acid (70:29:0.5 v/v). Therefore, the total phenol concentration could be determined directly from Folin assay that is widely used in the literature without ascorbic acid correction as in the case of our study. George et al. (49) showed that ascorbic acid exhibited a lower response than gallic acid (approx. 80% of the gallic acid absorbance at the same concentration). On the contrary, carotenoids apperared to exhibit a higher response value (app. 2–3 fold higher than that of gallic acid) that resulted in overestimation of the phenolic content if the extract was rich in carotenoids. But sour cherry is not a source of carotenoids and mainly contains anthocyanins. In the literature, the Folin-Ciocalteu method is commonly applied for the estimation of TPC by using a simple phenolic compound such as gallic acid as a standard and the results are reported as standard equivalent. As mentioned in the study of Box (50), the use of a simple component as a standard only provides an approximation and the results will be an underestimation due to presence of big phenolic compounds. The method can be applied to monitor the variations in concentrations of phenolic compounds in extracts rather than to determine absolute concentrations.

Phenolics in fruits are in both soluble and bound forms. Bound ones are mainly in the form of β-glycosides. In the literature there is a large variation in free phenol content. Vinson et al. (47) reported that 53% of total phenols were the bound ones for cherry with lower contribution to Folin assay. Sun et al. (51) showed that phenolics in fruits were mainly in soluble free form (92.3% for strawberry and 96.2% for cranberry). The same authors also reported that ascorbic acid only contributed 0% and 3% of the total antioxidant activity in cranberry (47 mg ascorbic acid/100 g fruit) and strawberry (257 mg/100 g fruit) for which the ascorbic acid content is higher than sour cherry (42 mg/g fruit). Another reason for the lower correlation can be the synergism among the free phenolic antioxidants in the extracts (47). The similar explanation was given for apple peel, flesh and whole apple extracts with 80% methanol (52).

CONCLUSIONS

By using elevated pressures, the extraction of phenolic compounds from fruit pomaces can be enhanced. HPE with ethanol gives recoveries higher than those obtained by SE with the same solvent. The results are even comparable with those obtained by SE with methanol. In the light of these findings, HPE can be a useful alternative for SE in terms of high efficiency, reduced extraction time and less amount of solvent. In comparison to ethanol extracts, different solvents can be tested for HPE. According to the TPC values, the extraction with carbon dioxide modified with ethanol gives a much lower yield of phenolic compounds than the other examined extraction methods. Further study will be the identification of the phenolic profile in the extracts obtained.

ACKNOWLEDGEMENTS

The authors thank Middle East Technical University- Research Projects Fund for supporting the project (BAP-2003-03-14-02 and BAP-2005-03-14-05). The financial support from The Scientific and Technological Research Council of Turkey (TÜBİTAK) for the project support (TOVAG-105O009) is gratefully acknowledged. Further thanks are extended to The Middle East Technical University Central Laboratory for the use of supercritical fluid extraction system.

REFERENCES

1. Schieber, A., Stintzing, F.C., and Carle, R. (2001) By-products of plant food processsing as a source of functional compounds-recent developments. *Trends Food Sci. Technol.*, 12 (11): 401.

2. Moure, A., Cruz, J.M., Franco, D.J., Dominguez, J.M., Sineiro, J., Dominguez, H., Nunez, M.J., and Parajo, J.C. (2001) Natural antioxidants from residual sources. *Food Chem.*, 72 (2): 145.
3. Peschel, W., Sanchez-Rabaneda, F., Diekmann, W., Plescher, A., Gartzia, I., Jimenez, D., Lamuela-Raventos, R., Buxaderas, S., and Codina, C. (2006) An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. *Food Chem.*, 97 (1): 137.
4. Balasundram, N., Sundram, K., and Samman, S. (2006) Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem.*, 99 (1): 191.
5. Gil, M.I., Tomás-Barberán, F.A., Hess-Pierce, B., and Kader, A.A. (2002) Antioxidant capacities, phenolic compounds, carotenoids, and vitamin C contents of nectarine, peach, and plum cultivars from California. *J. Agric. Food Chem.*, 50 (17): 4976.
6. Kähkönen, M., Hopia, A., Vuorela, H., Rauha, J.P., Pihlaja, K., Kujala, T.S., and Heinonen, M. (1999) Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.*, 47 (10): 3954.
7. Su, M.S. and Silva, J.L. (2006) Antioxidant activity, anthocyanins, and phenolics of rabbiteye blueberry (Vaccinium ashei) by-products as affected by fermentation. *Food Chem.*, 97 (3): 447.
8. Berardini, N., Knödler, M., Schieber, A., and Carle, R. (2005) Utilization of mango peels as a source of pectin and polyphenolics. *Innov. Food Sci. Emerging Technol.*, 6 (4): 442.
9. Bonilla, F., Mayen, M., Merida, J., and Medina, M. (1999) Extraction of phenolic compounds from red grape marc for use as food lipid antioxidants. *Food Chem.*, 66 (2): 209.
10. Rødtjer, A., Skibsted, L.H., and Andersen, M.L. (2006) Antioxidative and prooxidative effects of extracts made from cherry liqueur pomace. *Food Chem.*, 99 (1): 6.
11. Alonso-Salces, R.M., Korta, E., Barranco, A., Berrueta, L.A., Gallo, B., and Vicente, F. (2001) Pressurized liquid extraction for the determination of polyphenols in apple. *J. Chromotogr. A*, 933 (1–2): 37.
12. Escribano-Bailon, M.T. and Santos-Buelga, C. (2003) Polyphenol extraction from foods. In *Methods in Polyphenol Analysis*; Santos-Buelga, C. and Williamson, G. (eds.); RSC Publishing: London, pp. 1–16.
13. Zhang, S.Q., Junjie, Z., and Changzhen, W. (2004) Novel high pressure extraction technology. *Int. J. Pharm.*, 278 (2): 471.
14. Palma, M., Piñeiro, Z., and Barroso, C.G. (2001) Stability of phenolic compounds during extraction with superheated solvents. *J. Chromotogr. A*, 921 (2): 169.
15. Ju, Z.Y. and Howard, L.R. (2003) Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin. *J. Agric. Food Chem.*, 51 (18): 5207.
16. Palma, M. and Taylor, L.T. (1999) Extraction of polyphenolic compounds from grape seeds with near critical carbon dioxide. *J. Chromotogr. A*, 849 (1): 117.
17. Murga, R., Sanz, M.T., Beltrán, S., and Cabezas, J.L. (2002) Solubility of some phenolic compounds contained in grape seeds, in supercritical carbon dioxide. *J. Supercrit. Fluids*, 23 (2): 113.
18. Murga, R., Sanz, M.T., Beltrán, S., and Cabezas, J.L. (2003) Solubility of three hydroxycinnamic acids in supercritical carbon dioxide. *J. Supercrit. Fluids*, 27 (3): 239.

19. Cháfer, A., Fornari, T., Berna, A., and Stateva, R.P. (2004) Solubility of quercetin in supercritical CO_2 + ethanol as a modifier: Measurements and thermodynamic modelling. *J. Supercrit. Fluids*, 32 (1–3): 89.
20. Berna, A., Cháfer, A., Monton, J.B., and Subirats, S. (2001) High-pressure solubility data of system ethanol (1) + catechin (2) + CO_2(3). *J. Supercrit. Fluids*, 20 (2): 157.
21. Cháfer, A., Berna, A., Monton, J.B., and Muñoz, R. (2002) High-pressure solubility data of system ethanol (1) + epicatechin (2) + CO_2(3). *J. Supercrit. Fluids*, 24 (2): 103.
22. Berna, A., Cháfer, A., and Monton, J.B. (2001) High-pressure solubility data of the system resveratrol (3) + ethanol (2) + CO_2(1). *J. Supercrit. Fluids*, 19 (2): 133.
23. Chaovanalikit, A. and Wrolstad, R.E. (2004) Total anthocyanins and total phenolics of fresh and processed cherries and their antioxidant properties. *J. Food Sci.*, 69 (1): 67.
24. Wang, H.B., Nair, M.G., Strasburg, G.M., Booren, A.M., and Gray, J.I. (1999) Antioxidant polyphenols from tart cherries. *J. Agric. Food Chem.*, 47 (3): 840.
25. Wang, H.B., Nair, M.G., Strasburg, G.M., Booren, A.M., and Gray, J.I. (1999) Novel antioxidant compounds from tart cherries (Prunus cerasus). *J. Nat. Prod.*, 62 (1): 86.
26. Halvorsen, B.L., Holte, K., Myhrstad, M.C.W., Barikmo, I., Hvattum, E., Remberg, S.F., Wold, A.B., Haffner, K., Baugerod, H., Andersen, L.F., Moskaug, J.O., Jacobs, D.R., and Blomhoff, R. (2002) A systematic screening of total antioxidants in dietary plants. *J. Nutr.*, 132 (3): 461.
27. Blando, F., Gerardi, C., and Nicoletti, I. (2004) Sour cherry (Prunus cerasus L) anthocyanins as ingredients for functional foods. *J. Biomed. Biotechnol.*, 5: 253.
28. Thompson, D. (1982) Response surface experimentation. *J. Food Process. Pres.*, 6 (3): 155.
29. Caillet, S., Yu, H., Lessard, S., Lamoureux, G., Ajdukovic, D., and Lacroix, M. (2007) Fenton reaction applied for screening natural antioxidants. *Food Chem.*, 100 (2): 542.
30. Singleton, V.L. and Rossi, J.A. (1965) Colorimetry of total phenolics with phosphomolibdic phosphotungstic acid reagent. *Am. J. Enol. Viticult.*, 16: 144.
31. Brand-Williams, W., Cuvelier, M.E., and Berset, C. (1995) Use of a free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.*, 28 (1): 25.
32. Sánchez-Moreno, C., Larrauri, J.A., and Saura-Calixto, F. (1998) A procedure to measure the antiradical efficiency of polyphenols. *J. Sci. Food Agr.*, 76 (2): 270.
33. Mansouri, A., Embarek, G., Kokkalou, E., and Kefalas, P. (2005) Phenolic profile and antioxidant activity of the Algerian ripe date palm fruit (Phoenix dactylifera). *Food Chem.*, 89 (3): 411.
34. Atoui, A.K., Mansouri, A., Boskou, G., and Kefalas, P. (2005) Tea and herbal infusions, Their antioxidant activity and phenolic profile. *Food Chem.*, 89 (1): 27.
35. Kallithraka, S., Mohdaly, A.A., Makris, D.P., and Kefalas, P. (2005) Determination of major anthocyanin pigments in Hellenic native grape varieties (Vitis vinifera sp.). *J. Food Compos. Anal.*, 18 (5): 375.
36. Anagnostopoulou, M.A., Kefalas, P., Papageorgiou, V.P., Assimopoulou, A.N., and Boskou, D. (2006) Radical scavenging activity of various extracts and fractions of sweet orange peel (Citrus sinensis). *Food Chem.*, 94 (1): 19.
37. Richter, B.E., Jones, B.A., Ezzeli, J.L., Porter, N.L., Avdalovic, N., and Pohl, C. (1996) Accelerated solvent extraction: A technique for sample preparation. *Anal. Chem.*, 68 (6): 1033.

38. Cacace, J.E. and Mazza, G. (2003) Mass transfer process during extraction of phenolic compounds from milled berries. *J. Food Eng.*, 59 (4): 379.
39. Rostagno, M.A., Palma, M., and Barroso, C.G. (2004) Pressurized liquid extraction of isoflavones from soybeans. *Anal. Chim. Acta*, 522 (2): 169.
40. Kim, D.O., Heo, H.J., Kim, Y.J., Hyun, S.Y., and Chang, Y.L. (2005) Sweet and sour cherry phenolics and their protective effects on neuronal cells. *J. Agric. Food Chem.*, 53 (26): 9921.
41. Bastin, S. and Henken, R. (1997) Water content of fruits and vegetables. Available from University of Kentucky, Collage of Agriculture, Environmental and Natural Resource Issues Publications. www.ca.uky.edu/enri/pubs/enri129.pdf (accessed 5/8/2006).
42. Hamburger, M., Baumann, D., and Adler, S. (2004) Supercritical carbon dioxide extraction of selected medicinal plants-effects of high pressure and added ethanol on yield of extracted substances. *Phytochem. Anal.*, 15 (1): 46.
43. Özkal, S.G., Yener, M.E., and Bayındırlı, L. (2006) The solubility of apricot kernel oil in supercritical carbon dioxide. *Int. J. Food Sci. Technol.*, 41 (4): 399.
44. Roy, B.C., Goto, M., and Hirose, T. (1996) Extraction of ginger oil with supercritical carbon dioxide: experiments and modeling. *Ind. Eng. Chem. Res.*, 35 (2): 607.
45. Perva-Uzunalic, A., Skerget, M., Knez, Z., Weinreich, B., Otto, F., and Gruner, S. (2006) Extraction of active ingredients from green tea (Camellia sinensis): Extraction efficiency of major catechins and caffeine. *Food Chem.*, 96 (4): 597.
46. Cevallos-Casals, B.A., Byrne, D., Okie, W.R., and Cisneros-Zevallos, L. (2006) Selecting new peach and plum genotypes rich in phenolic compounds and enhanced functional properties. *Food Chem.*, 96 (2): 273.
47. Vinson, J.A., Su, X., Zubik, L., and Bose, P. (2001) Phenol antioxidant quantity and quality in foods: fruits. *J. Agric. Food Chem.*, 49 (11): 5315.
48. Asami, D.K., Hong, Y.-J., Barrett, D.M., and Mitchell, A.E. (2003) Comparison of the total phenolic and ascorbic acid content of freeze-dried and air-dried marionberry, strawberry, and corn grown using conventional, organic, and sustainable agricultural practices. *J. Agric. Food Chem.*, 51 (5): 1237.
49. George, S., Brat, P., Alter, P., and Amiot, J. (2005) Rapid determination of polyphenols and vitamin C in plant derived products. *J. Agric. Food Chem.*, 53 (5): 1370.
50. Box, J.D. (1983) Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substrates in natural water. *Water Res.*, 17 (5): 511.
51. Sun, J., Chu, Y.F., Wu, X., and Liu, R.H. (2002) Antioxidant and antiproliferative activities of common fruits. *J. Agric. Food Chem.*, 50 (25): 7449.
52. Pearson, D.A., Tan, C.H., German, J.B., Davis, P.A., and Gershwin, M.E. (1999) Apple juice inhibits human low density lipoprotein oxidation. *Life Sci.*, 64 (21): 1913.