Application of response surface methodology for the optimization of supercritical fluid extraction of essential oil from pomegranate (*Punica granatum* L.) peel

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Abstract Essential oils and volatile components of pomegranate (*Punica granatum* L.) peel of the Malas variety from Meybod, Iran, were extracted using supercritical fluid extraction (SFE) and hydro-distillation methods. The experimental parameters of SFE that is pressure, temperature, extraction time, and modifier (methanol) volume were optimized using a central composite design after a (2^{4-1}) fractional factorial design. Detailed chemical composition of the essential oils and volatile components obtained by hydro-distillation and optimum condition of the supercritical CO₂ extraction were analyzed by GC–MS, and seventy-three and forty-six compounds were identified according to their retention indices and mass spectra, respectively. The optimum SFE conditions were 350 atm pressure, 55 °C temperature, 30 min extraction time, and 150 µL methanol. Results showed that oleic acid, palmitic acid and (–)-Borneol were major compounds in both extracts. The optimum extraction yield was 1.18 % (w/w) for SFE and 0.21 % (v/w) for hydro-distillation.

Keywords Pomegranate (*Punica granatum* L.) peel · Essential oil · Supercritical fluid extraction (SFE) · Hydro-distillation (HD) · Response surface methodology (RSM)

Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest edible fruits, which is extensively cultivated around the world in many tropical and subtropical regions with different microclimatic zones (Salaheddin and Kader 1984). Pomegranate peels is the main waste fraction of pomegranate fruits and due to its therapeutic properties, it is widely used in the folk medicine of many cultures (Fadavi et al. 2006). Numerous studies have demonstrated that pomegranate peels contain many biologically active compounds including, natural antioxidants such as flavonoids and phenolic acids (Li et al. 2006; Singh et al. 2002) and substantial amounts of polyphenols such as ellagic tannins, ellagic acid and gallic acid (Nasr et al. 1996). Pomegranate peel extract delays the proliferation of human breast and prostate cancer cell lines (Toi et al. 2003). It is mentioned in Asian folk medicine, dried fruit peel is used for diarrhea and to treat respiratory and urinary tract infections. Also, pomegranate fruit peel exerted diverse pharmacological functions as anti-oxidant activity (Thring et al. 2009), anti-fertility effect (Gujral et al. 1960), cytotoxic activity (Sato 1990; Kulkarni et al. 2007), hepatoprotective activity (Murthy et al. 2002), and hypoglycemic activity (Hontecillas et al. 2009). Many studies have proven that the pomegranate peel extract has wound healing properties (Chidambara et al. 2004), immunomodulatory activity (Gracious et al. 2001), anti-bacterial activity (Prashanth et al. 2001), gastroprotective effect (Gharzouli et al. 1999), larvicidal activity (Morsy et al. 1998), anti-fungal activity (Dutta et al. 1998), anti-tumor action (Mavlyanov et al. 1997), anti-microbial effect (Navarro et al. 1996), anti-viral activity (Zhang et al. 1995), hypoglycemia effect (Nogueira and Pereira 1984; Nogueira and Pereira 1986), and anti-diabetic activity (Zofar and Singh 1990). In

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addition, pomegranate peel has been used in the preparation of cosmetics, tinctures, therapeutic formulae and food recipes (Nasr et al. 1996). The essential oils of plants have been usually isolated by either conventional hydro-distillation or solvent extraction techniques (Chryssavgi et al. 2008; Farah et al. 2006). The traditional methods for the isolation and purification of chemical components from plants tissues present some disadvantages. Overall, they require long extraction time, great solvent amounts, and sometimes have low efficiency. Furthermore, many natural products are thermally unstable and may degrade during thermal extraction or distillation. Existence of a few adjustable parameters for hydro-distillation and solvent extraction to control the selectivity of the process is another weakness of these methods. (Guan et al. 2007). Therefore, the necessity of a desirable extraction technique with high efficiency and selectivity is advised. Recently, the application of supercritical fluid extraction (SFE) has been widely studied for extracting oil from plant materials (Khajeh et al. 2010; Ara et al. 2014; Daneshvand et al. 2012; Ghasemi et al. 2011; Ara et al. 2015). Extraction processes using supercritical carbon dioxide are potential alternative methods for essential oil extraction to replace conventional extraction processes of expeller pressing and solvent extraction. Supercritical carbon dioxide not only has the higher extraction rate but also is non-toxic, non-explosive, non-flammable, cost-efficient, readily available, and easy to remove from the extracted materials (Gironi and Maschietti 2008). The goal of the present study was to extract essential oil compounds from pomegranate peel using response surface methodology (RSM) and to compare the results with that obtained by hydro-distillation technique.

Materials and methods

Reagents

HPLC grade acetone and methanol was purchased from Caledon (Georgetown, Ont., Canada). Carbon dioxide (99.99 % purity), contained in a cylinder with an eductor tube, was obtained from Roham Co. (Tehran, Iran).

Plant material and sample preparation

Ripened pomegranate from the Malas variety were obtained from Meybod (Yazd, Iran) in the middle of October 2012. Peels were separated manually and placed in a dark area and air-dried at room temperature for two weeks. Dried peels were grounded using a commercial grinder (Myson, China) producing particles with a mean diameter of 0.5 mm and stored at 4 °C.

Hydro-distillation

The pomegranate peels (100 g of dried material) were submitted to hydro-distillation for 4 h, using a Clevenger-type apparatus. The volatile distillate was collected over anhydrous sodium sulfate and refrigerated prior to analysis. The yield of the oil was 0.21 % (w/w), based on dry peel weight.

Supercritical fluid extraction procedure (SFE)

In order to fully explore the effects of SFE parameters, experiments were conducted in a laboratory-scale Suprex MPS/225 system (Pittsburgh, PA) in the SFE mode. Pomegranate peel powder (0.5 g) mixed well with 1 mm diameter glass beads, was loaded into a 3-mL high-pressure stainless steel extraction vessel, and filter mesh screens were placed at both ends of the vessel to prevent any carryover of particles. Liquid CO₂ was pumped into the vessel with a flow rate of approximately (0.4 ± 0.05 mL min⁻¹). The fluctuation of pressure and temperature were controlled to an accuracy of ±0.5 atm and ±0.5 °C, respectively. A Duraflow manual variable restrictor (Suprex) was used in the SFE system to collect the extracted analytes. The extracts were collected continuously from the outlet of the restrictor in a 3.0-mL volumetric flask containing acetone. The final volume of the extract was adjusted to 3.0 mL with acetone at the end of the extraction. For all the modifier studies, the modifier (methanol) was spiked directly into the sample in the extraction vessel before the extraction cell was attached to the SFE system. The amount of extracted oil was measured gravimetrically after the completion of each run, and then the extraction recovery percentage (%) was calculated as the percent ratio of the mass of extracted oil to the mass of pomegranate peel powder loaded into the extraction vessel.

Gas chromatography–mass spectrometry (GC/MS) analysis

GC–MS analyses were carried out using a Hewlett-Packard 5970 mass spectrometer operating in EI mode at 70 eV. The interface temperature was 250 °C; Mass range was from *m/z* 30–600 amu. The gas chromatograph was equipped with a 30 m DB-5 (5 % biphenyl + 95 % methylpolysiloxane) fused-silica capillary column, 0.53 mm i.d. and 1.5 µm film thickness, (Folsom, CA, USA). Helium (purity 99.999 %) was used as carrier gas with the flow rate of 0.8 mL min⁻¹. The temperatures of injector and detector were set at 250 and 300 °C, respectively. Oven temperature rise was programmed as follows: 50 °C for 5 min increased to 240 °C at 3 °C min⁻¹, held for 1 min, and finally rose at 15 °C min⁻¹ to 300 °C and

held for 3 min. The samples (1 μL) were injected with a split ratio of 1/10. The NIST MS 2.0 database and Wiley mass spectra libraries were searched and spectral matching of the components were performed. The data obtained were also confirmed by comparing their retention indices with those of authentic compounds.

Experimental design and statistical analysis

In order to minimize the number of experimental runs and time required, experimental design was adapted in accordance with the results of response surface methodology (RSM) for optimizing the essential oil extraction variables. A two-step optimization strategy, including a fractional factorial experimental design and a central composite design (CCD), was employed to optimize the experimental conditions providing the highest SFE recovery of *P. granatum* peel inside the experimental domain. In all cases, design generation and statistical analyses were performed by means of the statistical computer package “Design-Expert 7.0.0 Trial” (Stat-Ease Inc., Minneapolis).

Results and discussion

Screening of the effective parameters using fractional factorial design

Two-level fractional factorial design (2^{4-1}) with three replicates of center point and eleven experiments was performed in order to determine four factors including pressure (A), temperature (B), modifier (methanol) volume (C) and extraction time (D). It is important to maximize the contact of the supercritical fluid solvent with the sample material in order to enhance the extraction efficiency. SFE time and SFE mode can influence the solvent contact with sample material, (static with no follow-through or dynamic with follow-through). It was reported (Ara et al. 2014; Daneshvand et al. 2012) that, static extraction time improved the overall yield of the extraction. Therefore, according to preliminarily experiments the static time of 5 min was used for all the experiments. At the higher static extraction time of 5 min no improvements on the extraction yield were observed. Table 1 shows the main factors, their notations, and levels for half fractional factorial design. The standardized effect of the independent variables and their interactions on the dependent variables was investigated by pareto chart (Fig. 1). The vertical line on the plot judges the effects that are statistically significant. Moreover, a positive value (shown in dark blue) for the estimated effect indicates an increase in the extraction yield if the variable increases to its high level. A negative value (shown in pink) indicates that a better extraction

yield is obtained at low levels of the variables. The interpretation of this chart demonstrates that all experimental factors including, pressure, temperature, modifier volume, and extraction time have positive effect on the extraction yield in the range adopted for this study. Additionally, the pressure and extraction time factors are highly significant and an enhancement in their values increases the essential oil extraction yield. The modifier volume is a less significant factor and temperature has an insignificant effect.

Results of the half fractional factorial design demonstrated that pressure, extraction time and modifier volume require a final optimization. Therefore, a central composite design was carried out.

Central composite design (CCD)

This step is concerned with optimizing the values of pressure, extraction time and modifier volume in order to obtain the best response. In statistics, central composite design (CCD) is one of the most frequently used response surface designs. In this study, the CCD was used to optimize three important operating variables of SFE (pressure, modifier volume and extraction time) to achieve highest extraction yield. Table 2 shows the main factors, their notations, and levels for central composite design. The rotatable central composite design (RCCD) with $\alpha = 1.68$ has been carried out on seventeen randomized runs ($2^3 + (2 \times 3) + 3$), consists of a (2^3) factorial design augmented with (2×3) star points and 3 central points. ANOVA was used to select a suitable response surface model, the significances of the model equation and the model terms. Table 3 shows the ANOVA table for CCD design matrix. The *F*-value of 21.73 implies that the model is significant. Value of “Prob > *F*” less than 0.050 for a factor indicates that its effect is significant. Among the three independent variables tested, only pressure ($P < 0.050$) had a significant linear effect on the extraction yield, whilst the effects of modifier volume ($P = 0.0245$) and extraction time ($P = 0.008$) on essential oil yield were very small. A quadratic response surface model based on a higher *F*- and *R*-values and lower lack of fit (LOF) was selected. The response equation fitted the experimental

Table 1 Factors, factor notations, and their levels in half fractional factorial design

Factor	Notation	Level		
		−1	0	+1
Pressure (atm)	A	150	250	350
Temperature (°C)	B	35	45	55
Modifier volume (methanol) (μL)	C	0	50	100
Extraction time (min)	D	10	20	30

Fig. 1 Pareto chart of main effects obtained from 2^{4-1} fractional factorial design. The black horizontal line defines the 95 % confidence interval

Table 2 The experimental variables and levels of central composite design (CCD)

Factor	Notation	Level				
		$-\alpha^a$	-1	0	+1	$+\alpha$
Pressure (atm)	A	180	215	265	315	350
Modifier volume (μL)	B	50	70	100	130	150
Extraction time (min)	C	8	15	25	35	42

^a For this rotatable CCD, axial distance is 1.68

data with $R^2 = 0.96$, indicating that 96 % of the variability in the extraction yield of *P. granatum* peel essential oil can be explained by the model presented in a coded form in Eq. (1).

$$Y = 0.61 + 0.19A + 0.048B + 0.061C + 0.074 AC + 0.067A^2 - 0.065C^2 \quad (1)$$

This model consists of three main effects, a two-factor interaction, and two curvature effects. The normal probability graph and the histogram of residuals demonstrated that the residuals followed a normal distribution. The obtained regression models were used to calculate the response surface for each variable separately. Graphical optimization procedures were carried out for predicting the exact optimum level of independent variables leading

to the highest recovery of *P. granatum* peel essential oil during the SFE- CO_2 process. The Three-dimensional response surface (3D) plots were drawn to illustrate the interactive effects of the independent variables corresponded to the response variable. 3D surface plots of the pressure, modifier volume and extraction time were obtained by varying two variables within the experimental range and holding the other one constant at its central point. The response surfaces are shown in Figs. 2, 3 and 4. As noted from the statistical test for the regression models as discussed above, pressure had the most significant positive effect on the extraction yield than any other extraction parameters. The increase in essential oil yield with pressure can be explained by the increase in solvent power of supercritical CO_2 resulting from increments in density, which increases the solubility of the analytes (Brennecke 1997; Leitner 2000). The variation of temperature during the SFE affects the density and the volatility of the essential oil from the plant matrix. By increasing the temperature, the volatilities of the essential oil increases but the SFE density decreases. In this study, raising temperature to 55 °C increases the concentrations of content in the essential oil composition due to increasing the volatilities of the essential oil. Due to the limited solubility of polar organic compounds in the SFE, quantitative extraction of these compounds with pure supercritical carbon dioxide is impossible (Ekart et al.

Table 3 Analysis of variance (ANOVA) table of the quadratic response surface model

Source	SS	df	MS	F-value	Prob > F
Model	0.74	9	0.082	21.73	0.0003 (significant)
A–A	0.47	1	0.47	125.19	<0.0001
B–B	0.031	1	0.031	8.16	0.0245
C–C	0.051	1	0.051	13.37	0.0081
AB	0.0003125	1	0.0003125	0.082	0.7823
AC	0.044	1	0.044	11.49	0.0116
BC	0.0000125	1	0.0000125	0.0033	0.9558
A ²	0.051	1	0.051	13.42	0.008
B ²	0.0005587	1	0.0005587	0.15	0.7124
C ²	0.048	1	0.048	12.74	0.0091
Residual	0.027	7	0.003788		
Lack of fit	0.025	5	0.00509	9.54	0.0975 (not significant)
Pure error	0.001067	2	0.0005333		
Corrected total	0.77	16			

Fig. 2 3D-estimated response surface by plotting extraction yield (%) versus pressure (a) and modifier volume (b) at extraction time of 25 min at 55 °C

1999; Khajeh 2011). Modifier is added to an extraction process mainly for two reasons (Khajeh 2011): (1) to increase the polarity of the SFE in order to improve the solubility of the essential oil; and (2) to facilitate desorption of the essential oil from the plant matrix. The polar modifier molecules accelerate desorption processes by competing with the essential oil for the active binding sites; as well as by disrupting matrix structures. In the present work methanol was used as modifier, and it enhanced the solubility of solutes in SFE and, thus, the efficiency of extraction increased (Ekart et al. 1999; Khajeh et al. 2010; Ara et al. 2014). Concerning the description of the experimental data, the following conditions were selected to evaluate the performance of the extraction procedure: pressure of 350 atm, temperature of

55 °C, modifier volume of 150 μ L, and extraction time of 30 min.

Comparison of essential oil and volatile compounds extracted by SFE-CO₂ and hydro-distillation

The results of GC/MS analyses of *P. granatum* peel essential oil obtained by SFE and hydro-distillation were given in Table 4. The results show that different compounds can be extracted from *P. granatum* peel using SFE and hydro-distillation procedures. Table 4 indicates that the number of the essential oil and volatile compounds extracted by SFE (46 components) is lower than those obtained by hydro-distillation method (73 components). The percent of oleic acid

Fig. 3 3D-estimated response surface by plotting extraction yield (%) versus pressure (a) and extraction time (c) at modifier volume of 100 μ L at 55 $^{\circ}$ C

Fig. 4 3D-estimated response surface by plotting extraction yield (%) versus modifier volume (b) and extraction time (c) at pressure of 265 atm at 55 $^{\circ}$ C

(12.49 %), palmitic acid (11.65 %) and camphor (5.8 %) in the SFE was higher than hydro-distillation. The SFE extract exhibit higher proportion of compounds with higher molecular weight than the HD, indicating that the supercritical fluid extraction was more selective than the hydro-distillation method and required shorter extraction time. Furthermore, the SFE is less tedious and minimizes the risk of compound degradation due to heat. Additionally, the flexibility in

the management of the variables involved in the SFE process permitted us to optimize the experimental conditions, considering the selectivity of a substance or classes of substances of interest. This is shown in Fig. 5 where the abundance of volatile components obtained by the optimum condition provided by SFE (Pressure of 350 atm, temperature of 55 $^{\circ}$ C, Modifier volume of 150 μ L, and extraction time of 30 min) are plotted against time.

Table 4 Percentage of the volatile components of *P. granatum* peel obtained by different extraction process

Peak no.	Component	RI ^a (iu)	% HD ^b	% SFE ^c
1	Furfural	829	1.79	–
2	2-Hexenal	845	0.26	–
3	<i>Cis</i> -3-Hexen-1-ol	853	0.17	–
4	1-Hexanol	865	0.19	–
5	1-(2-furanyl)-ethanone	890	0.72	–
6	α -Pinene	926	0.42	1.38
7	Camphene	938	1.04	1.45
8	5-Methyl-furfural	953	1.3	–
9	β -Pinene	970	0.42	1.1
10	1-Octen-3-ol	981	0.23	–
11	6-Methyl-5-hepten-2-one	987	0.29	–
12	2-Pentyl-furan	994	0.21	–
13	Coumarone	1001	0.3	–
14	δ -3-Carene	1014	0.2	–
15	β -Cymene	1021	0.32	0.58
16	p-Cymene	1033	0.76	0.8
17	α -Limonene	1041	0.58	0.63
18	1,8-cineole	1047	3.14	2.86
19	Butylbenzene	1059	4.88	3.64
20	o-Guaiacol	1074	0.59	0.24
21	Linalool	1084	0.87	0.34
22	2-Methylbenzofuran	1093	1.4	–
23	Camphor	1128	3.05	5.8
24	Trans-carveol	1175	0.44	0.28
25	(–)-Borneol	1187	12.97	9.5
26	4-Terpineol	1196	0.46	0.2
27	Crypton	1203	0.96	–
28	α -Terpineol	1213	0.87	1.65
29	4,7-dimethylbenzofuran	1228	0.62	–
30	Trans-(+)-Carveol	1232	0.13	0.1
31	bornyl acetate	1245	0.69	1.1
32	Cuminal	1259	0.27	–
33	δ -Carvone	1262	0.35	–
34	Hexylbenzene	1271	1.05	0.54
35	2-Methoxyphenethyl alcohol	1292	1.25	–
36	Anethole	1302	0.3	0.1
37	α -bornyl acetate	1311	0.2	0.42
38	Cuminol	1320	0.63	–
39	Heptylbenzene	1345	0.94	–
40	Cerulignol	1358	0.16	–
41	Tetradecane	1386	0.22	0.83
42	β -Caryophyllene	1403	1.5	0.67
43	<i>Cis</i> - α -Bergamotene	1422	0.32	1.7

Table 4 continued

Peak no.	Component	RI ^a (iu)	% HD ^b	% SFE ^c
44	3,3,5,7-Tetramethyl-1-indanone	1447	0.43	–
45	trans-Geranyl acetone	1453	0.3	–
46	Phenylloctane	1468	0.64	0.23
47	Pentadecane	1495	0.18	0.25
48	Cadinene	1517	0.37	0.56
49	β -Caryophyllene oxide	1542	1.17	1.73
50	Lauric acid	1563	0.48	1.69
51	Nonylbenzene	1571	0.37	–
52	Hexadecane	1593	0.25	0.76
53	α -Cadinol	1612	1.13	1.8
54	Decylbenzene	1631	0.23	–
55	Cyclotetradecane	1643	0.64	–
56	Heptadecane	1692	0.38	1.3
57	Myristic acid	1762	0.53	1.58
58	Octadecane	1812	0.38	1.25
59	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	1854	0.57	0.2
60	Palmitonitrile	1873	0.27	–
61	Nonadecane	1900	0.38	1.54
62	Phthalic acid, butyl 8-methylnonyl ester	1920	0.26	–
63	Pentadecanoic acid, 14-methyl-, methyl ester	1936	0.39	–
64	Dibutyl phthalate	1957	10.82	4.43
65	Palmitic acid	1976	9.86	11.65
66	Eicosane	2012	0.31	1.4
67	Heneicosane	2098	0.39	1.73
68	Oleic acid	2114	11.65	12.49
69	Steric acid	2132	0.49	1.43
70	Docosane	2159	0.82	3.5
71	Tricosane			
	2298	0.51	2.86	
72	Octacosane	2607	0.26	2.53
73	Nonacosane			
	2812	1.94	2.74	
74	Triacotane	2945	–	1.72
	Total	–	93.86	95.28

Hydro-distillation: 4 h; supercritical CO₂ extraction: 55 °C, 350 atm, 30 min, and modifier (methanol) volume 150 μ L

^a RI, Kovat's retention index using a DB-5 MS-column

^b %, Relative area percent (peak area relative to the total peak area) for hydro-distillation

^c %, Relative area percent (peak area relative to the total peak area) for supercritical fluid extraction

Fig. 5 The GC–MS chromatogram obtained by SFE in the optimum condition. *Numbers* are related to the components in Table 4

Conclusions

Pressure had the greatest impact on the extraction yield of pomegranate volatile components. The highest yield of 1.18 % was obtained at a pressure of 350 atm, temperature of 55 °C, modifier (methanol) volume of 150 µL, and extraction time of 30 min. SFE showed different results in comparison with the hydro-distillation procedure. The SFE method offers many important advantages over hydro-distillation. SFE requires shorter extraction time (30 min vs. 4 h for hydro-distillation).

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