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ORIGINAL PAPER

Optimization of Supercritical Carbon Dioxide Extraction of Bioactive Flavonoid Compounds from Spearmint (*Mentha spicata* L.) Leaves by Using Response Surface Methodology

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Abstract The bioactive flavonoid compounds of spearmint (*Mentha spicata* L.) leaves were obtained by using supercritical carbon dioxide (SC-CO_2) extraction. Extraction was carried out according to face-centred central composite design, and independent variables were pressure (100, 200 and 300 bar), temperature (40, 50 and 60 °C) and co-solvent amount (3, 6 and 9 g/min). The extraction process was optimized by using response surface methodology for the highest crude extraction yield of bioactive flavonoid compounds. The optimal conditions were identified as 209.39 bar pressure, 50.00 °C temperature and 7.39 g/min co-solvent amount. The obtained extract under optimum SC-CO_2 condition was analysed by high-performance liquid chromatography. Seven bioactive flavonoids including catechin, epicatechin, rutin, luteolin, myricetin, apigenin and naringenin were identified as major compounds. The results of quantification showed that spearmint leaves are potential source of antioxidant compounds.

Keywords Spearmint (*Mentha spicata* L.) · Supercritical carbon dioxide (SC-CO_2) extraction · Response surface methodology · Bioactive flavonoid compounds · HPLC

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Introduction

Spearmint (*Mentha spicata* L.) belongs to the genus *Mentha* in the family *Labiatae* (*Lamiaceae*). It is usually known as 'Pudina' in most Indian-language countries. Spearmint (*M. spicata* L.) has post-digestive effect and hot potency, and its taste is pungent (Bimakr et al. 2008; Sweetie et al. 2007). Rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), thyme (*Thymus vulgaris* L.) and lavender (*Lavandula angustifolia Mill.*) are native to the Mediterranean region; balm (*Melissa officinalis* L.) and spearmint (*M. spicata* L.) are common plants in Britain and other European countries (Wang et al. 2004; Paranjpe 2001).

Several researchers pointed out that plants which belong to the *Lamiaceae* family are a source of compounds having high antioxidant, anti-inflammatory, antiallergy and anti-depression activity (Choudhury et al. 2006; Gopalan et al. 1999; Ali et al. 2002; Zheng and Wang 2001; Liu and Zhu 2007). Flavonoids which are widely distributed in the leaves, seeds, bark and flowers of plants are a broad class of low molecular weight compounds. Flavonoids are highly

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effective antioxidant and less toxic than synthetic antioxidants, such as butylated hydroxytoluene, butylated hydroxyanisole and propyl gallate (Erkan et al. 2008). Therefore, extraction of flavonoid compounds from different herb materials has received great attention (Kahkonen et al. 1999; Heim et al. 2002), and it is interesting to find an effective method to extract flavonoids from spearmint leaves.

Supercritical fluid extraction (SFE) was developed in 1960 (Lin et al. 1999). In recent years, many studies have been done to use supercritical fluid extraction with carbon dioxide (CO_2) as a solvent for extraction of natural compounds from different raw materials (Baysal et al. 2000; Lang and Wai 2001; Diaz-Maroto et al. 2002; Hu et al. 2007). The combined liquid-like solvating capabilities and gas-like transport properties of supercritical fluids make them particularly suitable for extracting bioactive compounds from plant tissues with a high degree of recovery in a short period of time. It was believed that, by using SFE, the extraction time can be reduced to tens of minutes compared with that by liquid–solid extraction that required hours or days (Wheeler and McNally 1989). Moreover, the solvent strength of supercritical fluid can be manipulated by changing pressure (*P*) and/or temperature (*T*); therefore, it may achieve a remarkably high selectivity (Liza et al. 2009; Reverchon et al. 1993).

The special note of this process for selective extraction of soluble compounds from a raw material is usage of gases above their critical points (Reverchon 1997; Baysal et al. 2000; Daood et al. 1999; Diaz-Maroto et al. 2002).

Carbon dioxide (CO_2) is an inert, non-toxic, environmentally friendly solvent and allows SFE at temperatures near room temperature and relatively low pressures (Liu et al. 2009; Leal et al. 2008; Ueno et al. 2008). However, CO_2 is not a suitable solvent for extraction of polar compounds because of its non-polar nature. This is the main limiting step in its use to separate polar bioactive compounds such as flavonoids. To increase the polarity of extraction solvent, some food-grade solvents like ethanol can be used as co-solvent (Lang and Wai 2001). Food industry is a major user of the supercritical carbon dioxide (SC-CO_2) extraction process for extraction and fractionation of fats, oils, essences, pigments and functional or bioactive compounds (Mitra et al. 2009).

There are many variables in the SFE process that may affect the efficiency of the extraction such as pressure, time, temperature, solvent flow rate, co-solvent, co-solvent flow rate, particle size, porosity, density, bed diameter and height. Optimization of the experimental conditions is a critical step in developing a successful SC-CO_2 process because of the effect of various variables on the extraction efficiency. The response surface methodology (RSM) has been demonstrated to be a powerful tool for determining the factor effects and their interactions, which allows process optimization to be conducted effectively (Bas and Boyaci 2007; Zarena et al. 2010). This method is preferred to experimental design for fitting polynomial model to analyse the response surface of multi-factor combinations. RSM is a faster and more economical method for gathering research results than classic one-variable-at-a-time or full-factor experimentation (Liu et al. 2009).

It was shown that the spearmint extracts has a high potential antioxidant activity (Sweetie et al. 2007). Therefore, it is interesting and useful to find an optimum condition to obtain the highest crude extraction yield of bioactive compounds from spearmint leaves. The aim of this work was to study and optimize the effect of supercritical carbon dioxide extraction parameters, namely pressure, temperature and co-solvent flow rate, on the crude extraction yield of bioactive compounds through the RSM. Then, the bioactive flavonoid compound profile of the obtained extract under optimal condition was analysed by using high-performance liquid chromatography (HPLC) to identify and quantify the major bioactive flavonoid compounds.

Material and Methods

Material and Reagents

The leaves of spearmint (*M. spicata* L.) were obtained from Cameron Highland in Pahang, Malaysia. After harvesting, the leaves were separated and washed under tap water. Leaves were dried at 40 °C in a ventilated drying oven (1350FX, USA) for 24 h and then stored at ambient temperature (22 °C) in the dark. The samples were ground in a grinding mill (MX- 335, Panasonic, Malaysia) for 10 s to produce a powder with an approximate size of 0.525 mm.

Carbone dioxide (CO_2, SFE grade), contained in a dip tube cylinder, was purchased from the MOX Company in Malaysia. Methanol (MeOH, HPLC grade) and ethanol (99.9%, analytical grade) were purchased from Scharlau Chemical, European Union. Trifluoroacetic acid (TFA, 98%) was obtained from Sigma Aldrich, Germany. All flavonoid standards including (+)-catechin, (−)-epicatechin, apigenin, rutin, luteolin, kaempferol, myricetin and naringenin were purchased from Sigma Aldrich, Germany.

Supercritical Carbon Dioxide Extraction

SC-CO_2 extraction was performed on a supercritical fluid extractor (ABRP200, Pittsburgh, PA, USA) with an extractor volume of 500 ml. The schematic diagram of SC-CO_2 extractor unit was shown in Fig. 1. The flow rate of CO_2, the extraction temperature and pressure were adjusted by the ICE software, and the extraction time was

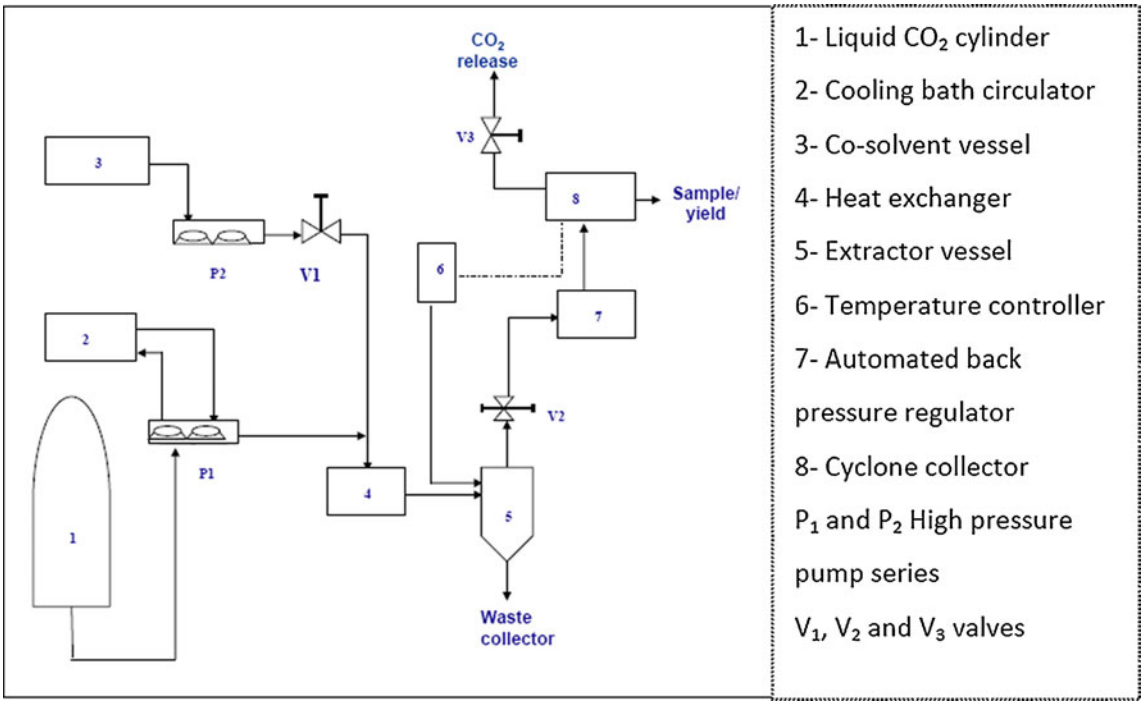

Fig. 1 Schematic diagram of supercritical fluid extractor

measured by the stopwatch. Liquid CO_2 was supplied from a gas cylinder. Before liquid CO_2 passed into the extraction vessel, filled with the samples, by means of a pump (P-50, Thar Designs, Inc. Pittsburg, PA, USA), it was pressurized to the desired pressure and heated to the specified temperature in order to reach the supercritical state.

Ethanol (99.9% purity) acted as the co-solvent to increase the polarity of solvent and improve the efficiency of SC-CO_2 extraction of bioactive compounds from spearmint leaves. During SFE, the extraction vessel containing raw plant material is equipped with temperature controllers and pressure valves at both inlet and outlet to keep desired extraction conditions. The fluid pump pressurized the extraction vessel. In cyclone, the solvating power of the fluid is decreased by decreasing the pressure or increasing the temperature of the fluid.

The crude extraction yield of bioactive compounds was then measured gravimetrically and reported as follows:

$$Y = \frac{m_{\text{extract}}}{m_{\text{herb}}} \times 1,000$$

where Y is the crude extraction yield (mg/g), m_{extract} is the crude extract mass (g) and m_{herb} is the extracted herb mass (g).

High-Performance Liquid Chromatography Analysis

The bioactive flavonoid compounds of the spearmint leaves extracts were analysed by HPLC method (Wang and Helliwell 2001). The HPLC analyses were performed with a Water 600 pump Controller and 9486 tunable absorbance UV detector and equipped with an Eclipse XDRC18 reversed-phase column (25 cm×4.6 mm×5 μm, Supelco, USA). Classic Millennium 2010 software was used for manipulation of data processing. Classic Millennium 2010 software was used for manipulation of data processing. The mobile phase was composed of TFA in deionized water at pH 2.5 (solvent A) and pure HPLC-grade methanol (solvent B). Gradient chromatography was run at 1.0 ml/min as follows: 0–30 min, solvent B increasing linearly from 0% to 60%; 30–35 min, solvent B increase to 100%; 35–40 min, solvent B decrease to 0%. The volume of the injection loop was 20 μl. A 70% MeOH was also required for washing the system. The temperature was set to room temperature, and the flavonoids were detected at 280 nm. Quantity calculations were made according to the linear calibration curves of standards.

Experimental Design

Response surface methodology was employed to optimize the operating conditions of the SFE process with three independent variables, namely temperature (40–60 °C, X_1), pressure (100–300 bar, X_2) and co-solvent flow rate (3–9 g/min, X_3), to obtain the highest crude extraction yield (Table 1). A face-centred central composite design (CCD-FC) was used for designing the experimental data. This design generated 20 treatments with six replications at the

Table 1 Levels of independent variables established according to the CCD-FC

Independent variables	Independent variables level		
	−1	0	+1
Temperature, X_1 (°C)	40	50	60
Pressure, X_2 (bar)	100	200	300
Co-solvent flow rate, X_3 (g/min)	3	6	9

centre point to estimate the repeatability of the method (Table 2). Based on preliminary studies, the supercritical CO_2 flow rate was maintained at 15 g/min, and the duration of static and dynamic extraction time was fixed at 30 and 60 min, respectively. The powdered plant material (30 g) was mixed with 90-g glass beads (2.0 mm in diameter), placed into the extractor vessel. All experiments were carried out in a randomized order to minimize the effect of unexplained variability in the observed response due to extraneous factors. Experimental design, data analysis and quadratic model building were conducted using the software Minitab v. 14.

Table 2 Face-centred central composite design of three independent variables and response for the crude extraction yield of spearmint leaves

Experiment no.[a]	Independent variables			
	Temperature (°C)	Pressure (bar)	Co-solvent flow rate (g/min)	Experimental value[b]
1	40	100	3	30.213
2	60	100	3	45.600
3	40	300	3	43.666
4	60	300	3	51.540
5	40	100	9	41.375
6	60	100	9	53.212
7	40	300	9	50.070
8	60	300	9	53.761
9 (C)	50	200	6	66.132
10 (C)	50	200	6	67.468
11 (C)	50	200	6	66.770
12 (C)	50	200	6	66.321
13	40	200	6	54.513
14	60	200	6	68.167
15	50	100	6	48.710
16	50	300	6	56.375
17	50	200	3	59.912
18	50	200	9	68.118
19 (C)	50	200	6	68.456
20 (C)	50	200	6	67.211

C centre point

[a] Experiments were performed in random order

[b] $Y = \frac{m_{\text{extract}}}{m_{\text{herb}}} \times 1,000$

Response Surface Modelling

A multiple-regression model (a polynomial equation of second order) was assumed for predicting Y variable. Regression equation and analysis of variance (ANOVA) were conducted for determining regression coefficients with statistical significance of model terms and for fitting the mathematical models to the experimental data. The generalized polynomial model proposed for predicting the response variables as a function of independent variables is given as

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

where Y_i is the predicted response; β_0 is the offset term; β_1, β_2 and β_3 are the regression coefficients for linear effect terms; β_{11}, β_{22} and β_{33} are the quadratic effects; and β_{12}, β_{13} and β_{23} are the interaction effects. In this model, X_1, X_2 and X_3 are the independent variables.

Table 3 Regression coefficients of the final regression model and ANOVA for the crude extraction yield in the CCD-FC design

Source	Coefficient	Sum of squares	*df*	Mean squares	*F* ratio	*p* value
X_1	6.000	275.3	1	275.3	206.84	0.000*
X_2	0.707	131.78	1	131.78	99.11	0.000*
X_3	6.597	126.77	1	126.77	95.34	0.001*
X_1^2	−0.049	64.59	1	64.59	48.58	0.000*
X_2^2	−0.001	511.93	1	511.93	385.01	0.000*
X_3^2	−0.246	12.97	1	12.97	9.75	0.015*
X_1X_2	−0.002	30.65	1	30.65	23.05	0.001*
X_1X_3	−0.032	7.47	1	7.47	5.62	0.049*
X_2X_3	−0.004	12.88	1	12.88	9.68	0.016*
Lack of fit		9.67	5	1.93	2.66	0.074
Pure error		3.63	5	0.73		
Total		2,307.32	19			
R^2	0.994					
R_{adj}^2	0.998					

X_1, X_2 and X_3 are the main effects of temperature (°C), pressure (bar) and co-solvent flow rate (g/min), respectively. X_1^2, X_2^2 and X_3^2 are the quadratic effects of temperature (°C), pressure (bar) and co-solvent flow rate (g/min), respectively. X_1X_2, X_1X_3 and X_2X_3 are the interaction effects of temperature (°C) and pressure (bar); the interaction effect of temperature (°C) and co-solvent flow rate (g/min); and the interaction effect of pressure (bar) and co-solvent flow rate (g/min), respectively

*$p<0.05$ (significant at this level)

The significance of the equation parameters for the response variable was assessed by the probability (p) of 0.05. The statistically found non-significant ($p>0.05$) terms were dropped from the initial models, and the experimental data were refitted only to significant ($p<0.05$) parameters to obtain the final reduced model.

Results and Discussion

Response Surface Analysis

The results of different runs are shown in Table 2. An ANOVA was conducted to determine the significant effects of process variables on the response. The estimated regression coefficients of three independent variables, along with the corresponding R^2, $R_{adj}{}^2$, p values and lack-of-fit test for the reduced response surface model, were displayed in Table 3. A high R^2 (0.994) indicates that the data fitted satisfactorily to the second-order polynomial reduced model. Thus, more than 99% of the response variation could be accurately explained as the function of three SFE process parameters.

Model Fitting

The mathematical model representing the extraction yield of spearmint as a function of the independent variables within the region under investigation was expressed by the following equation:

$$Y = -194.03 + 6.00X_1 + 0.707X_2 + 6.597X_3 - 0.049X_1^2 - 0.001X_2^2 - 0.246X_3^2 - 0.002X_1X_2 - 0.032X_1X_3 - 0.004X_2X_3$$

where Y is the extraction yield of spearmint and X_1, X_2 and X_3 are the uncoded variables for temperature, pressure and co-solvent amount, respectively.

In general, exploration and optimization of a fitted response surface may produce poor or misleading results, unless the model exhibits a good fit, which makes checking the model adequacy essential (Liyana-Pathirana and Shahidi 2005; Scavroni et al. 2005). The p value of the model was less than 0.05 (p value<0.05), which indicated that the model fitness was significant (Table 3). Meanwhile, the lack-of-fit value of the model was non-significant (0.074), indicating that the model equation was adequate for predicting the crude extraction yield under any combination of values of the variables (Table 3).

Coefficient (R^2) of determination is defined as the ratio of the explained variation to the total variation and is a measurement of the degree of fitness (Wang et al. 2008). A small value of R^2 indicates a poor relevance of the dependent variables in the model (Sin et al. 2006). By analysis of variance, the R^2 value of this model was determined to be 0.994, which showed that the regression model defined well the true behaviour of the system.

Numerical optimization was carried out by using response optimizer to predict the optimal condition in order to obtain the highest crude extraction yield of spearmint

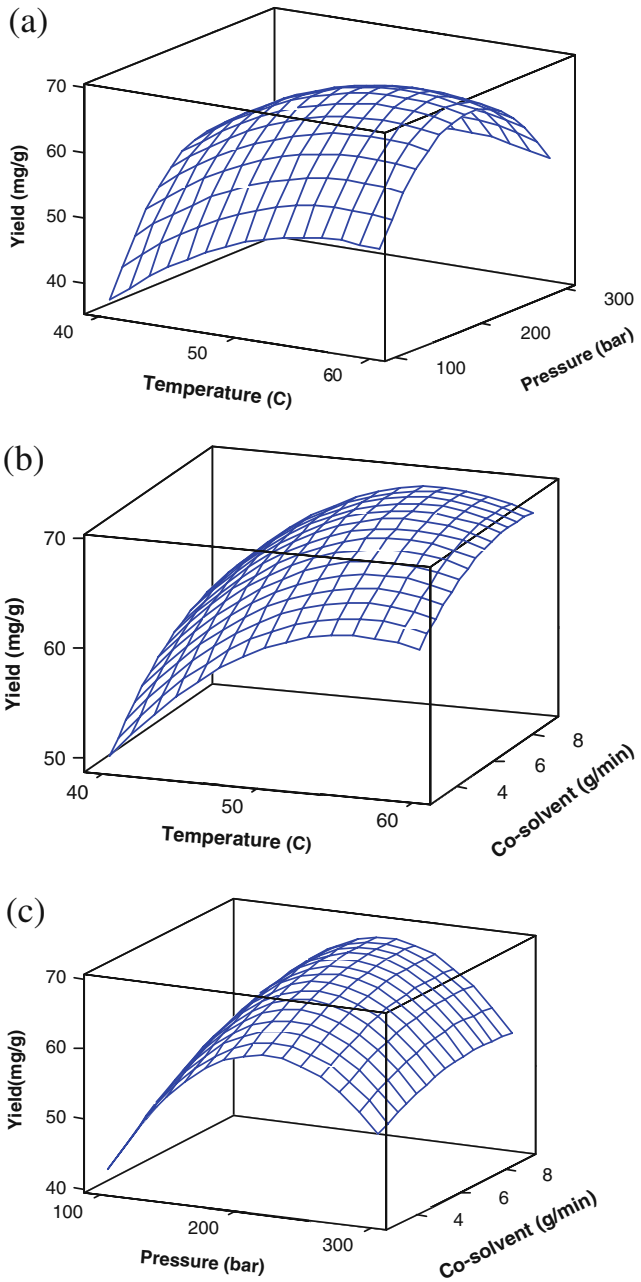

Fig. 2 Response surface plots: **a** yield vs. extraction temperature and pressure at constant co-solvent amount of 6 g/min; **b** yield vs. extraction temperature and co-solvent amount at constant pressure of 200 bar; **c** yield vs. extraction pressure and co-solvent amount at constant temperature of 50°C

leaves. The optimum conditions were identified as 209.31 bar pressure, 50.00 °C temperature and 7.39 g/min co-solvent flow rate. After extraction under these optimal conditions, the extraction yield of spearmint was 67.86±0.32 mg/g, and according to the Tukey test, this value was not significantly different to the predicted value (68. 09 mg/g) at the 95% confidence interval.

Effects of Temperature, Pressure and Co-solvent Amount on the Extraction Yield

The effects of pressure, temperature and co-solvent flow rate on the crude extraction yield of bioactive compounds from spearmint leaves, as well as their interactions, are shown in Fig. 2. An increasing co-solvent amount resulted in a higher extraction yield, while the crude extraction yield reached a maximum when co-solvent amount was up to a certain value, with no further significant improvement (Fig. 2b, c). In another study which was conducted by Wang et al. (2008), they found a similar result about the effect of co-solvent flow rate on the flavonoid yield from *Pueraria lobata*. This could be explained by the fact that the polar constituents in the plant would be easier to extract with a more polar solvent. As shown in Fig. 2a, c, there was an optimal value for pressure to obtain the highest extraction yield of bioactive compounds. A value lower or higher than this will lead to a decrease in extraction yield. This optimal value for pressure could vary when different temperatures and co-solvent amounts were employed.

At constant temperature, increasing the pressure will increase the density of theSC-CO_2. The solvent strength of SC-CO_2 increases with the density of CO_2. As the density increased, the distance between the molecules decreased; therefore, the interaction between the analytes and CO_2 increased, leading to greater solubility of the analytes in CO_2 (Liza et al. 2009). Therefore, the increase in pressure will also accelerate mass transfer analytes and solvent in the supercritical extractor vessel system and improve the extraction yield. This suggests that the solubility of matrix compounds in SC-CO_2 is proportional to the density of SC-CO_2. This result was clearly shown in higher temperature at 50 and 60 °C. In this study, the extraction yield increased with increasing pressure from 100 to 200 bar, which was caused by the increased supercritical CO_2 density at higher pressures. However, an increase in the pressure level (above 200 bar) led to an unexpected reduction in the extraction yield. In agreement with a previous study (Rezaei and Temelli 2000), it was pointed out that at higher pressure the diffusion rates of solutes are reduced which may be related to the supercritical fluid medium. Therefore, at a pressure above 200 bar, less extraction efficiency than that expected by the solubility enhancements at higher pressures (above 200 bar) was obtained as a result of the key role of diffusion in the mass transfer rates of the extractable materials from the sample matrix into the supercritical fluid environment (Rezaei and Temelli 2000). Similar results were obtained by Liza et al. (2009) and Wang et al. (2008) in their researches. In their investigations, it had been confirmed that, to obtain the highest yield from herb matrix, there was an optimal value for pressure (Liza et al. 2009; Wang et al. 2008).

Figure 2a, b shows the effect of temperature on the extraction yield of spearmint (*M. spicata* L.) leaves in SC-CO_2 extraction at three temperature levels of 40, 50 and60 °C. Temperature had a similar effect on the extraction yield to co-solvent. The extraction yield was increased with increasing temperature until 50 °C, after which the extraction yield was reduced due to thermosensitivity of flavonoid compounds (Nilufer et al. 2009). The effect of temperature on the crude extraction yield of spearmint leaves was in agreement with those found by Wang et al. (2008) on flavonoid yield from *P. lobata* (Wang et al. 2008). The density of CO_2 at constant pressure is reduced with increasing temperature, leading to reduction of the solvent power of supercritical CO_2. The effect of temperature on solute solubility is different at pressures in the critical range. Near the critical

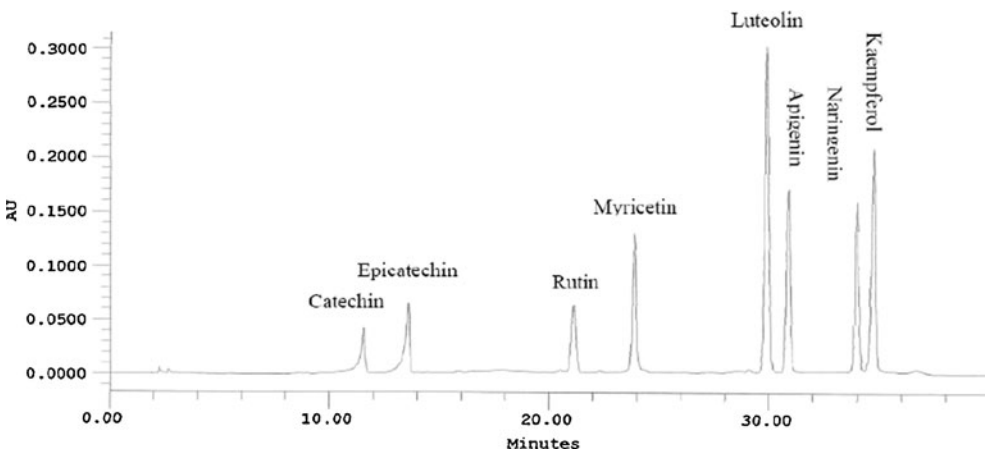

Fig. 3 HPLC chromatograms of mix standard

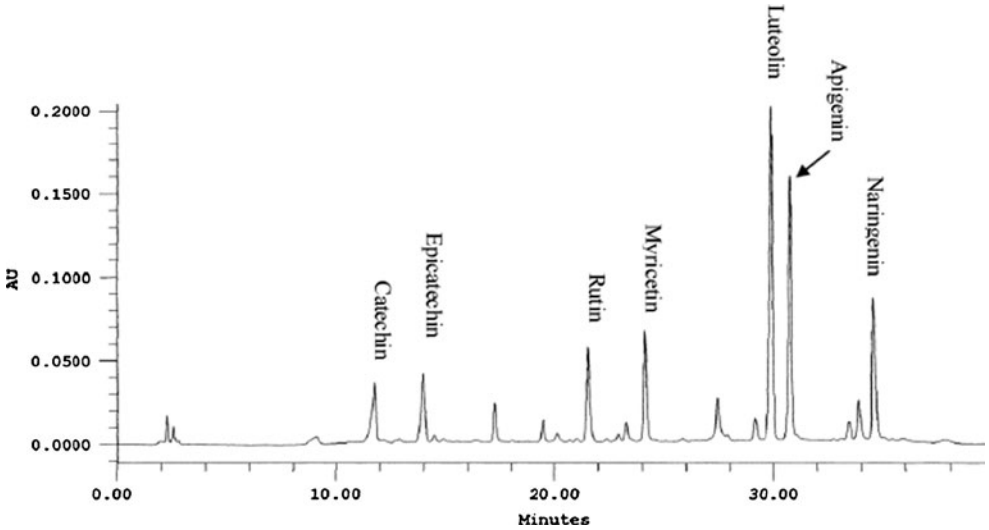

Fig. 4 HPLC chromatogram of optimum SC-CO_2 extraction condition

pressure (P_c=72.8 atm), the fluid density is very sensitive to temperature (Lang and Wai 2001). This might be the reason that crude extraction yield was changed significantly when temperature was changed over the range of 40−60 °C. A moderate increase in temperature can lead to a large decrease in fluid density, with a consequent reduction in solute solubility (Roop et al. 1989). However, the increase in temperature will also accelerate mass transfer and improve the extraction yield (Wang et al. 2008). The increase of temperature can increase the vapour pressure of the extractable compounds. Thus, the tendency of the compounds to be extracted is increased to pass in the supercritical fluid phase (Reverchon and De Marco 2006). For a volatile solute, there is competition between its solubility in supercritical carbon dioxide and its volatility (Pourmortazavi and Hajimirsadeghi 2007). Therefore, it is difficult to predict the effect of temperature. In the present study, the extraction yield increased with temperature; in this manner, the solute vapour pressure played a key role leading to an increase in the extraction yield. The main requirement of a co-solvent is that it is a good solvent in its liquid state for the target analyte. The co-solvent improves supercritical carbon dioxide extractability by increasing the polarity of the carbon dioxide. Choi et al. (1999) have observed that the addition of methanol can drastically increase the extraction yield of hyoscyamine and scopolamine from plant matrices. In this study, a similar effect was shown for ethanol. It increased the polarity of the extraction fluid and greatly improved the extraction yield of spearmint leaves (Choi et al. 1999).

Identification and Quantification of the Extracted Compounds

The extract obtained under optimum SC-CO_2 treatment conditions (209.31 bar, a temperature of 50.00 °C and 7.39 g/min co-solvent) was analysed by HPLC to identify and quantify major bioactive flavonoid compounds. The obtained extracts under the minimum and maximum levels of each studied parameters were taken as control. Figures 3 and 4 illustrated the separation of a standard mixture of flavonoids and experimental extract, respectively. Detailed identification and quantification of the compounds extracted by SC-CO_2 under different conditions were presented in Table 4.

Table 4 Identification and quantification of the compounds extracted by SC-CO_2 under different conditions

Experimental condition	Flavonoid content (mg/g)						
	Catechin	Epicatechin	Rutin	Myricetin	Luteolin	Apigenin	Naringenin
Type 1[a]	0.081	0.140	0.122	–	0.070	0.373	–
Type 2[b]	0.135	0.150	0.156	0.182	0.648	0.459	0.267
Type 3[c]	0.130	0.146	0.134	0.171	0.634	0.438	0.243

[a] Minimum level of each studied parameter (40 °C, 100 bar and 3 g/min co-solvent)

[b] Optimum level of each studied parameter (50 °C, 209.31 bar and 7.39 g/min co-solvent)

[c] Maximum level of each studied parameter (60 °C, 300 bar and 9 g/min co-solvent)

As it was mentioned in Table 4, under the minimum level of each parameter, five flavonoid compounds including (+)-catechin, (−)-epicatechin, rutin, luteolin and apigenin were extracted with lower concentration. However, with the use of the optimum conditions, seven bioactive flavonoids including (+)-catechin, (−)-epicatechin, rutin, myricetin, luteolin, apigenin and naringenin were detected with good separation and high concentration. In the product obtained under optimum conditions, luteolin (0.648 mg/g) had the highest concentration among the other flavonoids. When using the maximum level of studied factors, the concentration of flavonoids was reduced compared with that when using the optimum values. The volatility of the studied compounds can cause this unexpected result.

Based on the data obtained, the SC-CO_2 extraction is an effective method for extraction of bioactive compounds from spearmint leaves. Other researchers have reported a positive correlation between antioxidant activity and flavonoid compounds (Wangensteen et al. 2004; Zheng and Wang 2001). Flavonoids, a broad class of phenolic compounds, are effective hydrogen donors, which make them good antioxidants (Sweetie et al. 2007). Adding ethanol as co-solvent can improve the solubility of bioactive flavonoid compounds in CO_2 (Hu et al. 2007).

Conclusion

The high correlation of the mathematical model indicated that a quadratic polynomial model could be employed to optimize extraction yield from spearmint by supercritical carbon dioxide extraction. From the response surface plots, three factors (pressure, temperature andco-solvent flow rate) significantly influenced the crude extraction yield independently. The optimal conditions to obtain the highest crude extraction yield of spearmint were determined to be 209.31 bar, 50.00 °C and 7.39 g/min of co-solvent. Under the optimal conditions, the difference between experimental value (67.86±0.32 mg/g) and predicted value (68.09 mg/g) was small. Thus, this methodology could provide a basis for a model to examine the non-linear nature between independent variables and response in a short-term experiment. Also, by considering the HPLC analysis, the spearmint leaves are a potential source of antioxidant compounds. Plant sources like spearmint leaves may bring new natural products into the food industry with safer and better antioxidants that provide good protection against oxidative damage, which occurs both in the body and in the processed food.

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