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Berberis Vulgaris Fruit: Determination of Phenolic Compounds in Extracts Obtained by Supercritical CO_2 and Soxhlet Methods Using HPLC

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Abstract

Recently, research studies on nutraceutically important polyphenolic substances have attracted intensive attention. *Berberis vulgaris* is an important source of polyphenolic compounds and is often used in traditional medicine. In this study, the extraction of rutin and apigenin rich oil from *Berberis vulgaris* fruits was evaluated by supercritical carbon dioxide (Sc-CO_2) extraction method with and without co-solvent. As valuable antioxidants, rutin and apigenin content of extracts were analyzed by HPLC, and their amounts were maximized via parametric optimization. The rutin compound studied in this research has the potential to be a drug against the COVID-19 virus. The operating conditions were considered in the range of 35–70 °C temperatures, 140–240 bar of pressures, 0.35–1.00 mm of mean particle sizes, 3–7 l/min of CO_2 flow rates, and 0–8% w/w co-solvent. As a result of Sc-CO_2 extractions, the amounts of rutin and apigenin were found as 173 ± 14.97 µg/g and 2.91 ± 0.11 µg/g, respectively, with the 8% (w/w) co-solvent addition. The amounts of rutin and apigenin obtained by Soxhlet extractions were found as 208.81 ± 8.48 µg/g and 6.55 ± 0.21 µg/g, respectively. When the Sc-CO_2 method was compared with the Soxhlet method, it was seen that the fast and eco-friendly Sc-CO_2 method was an ideal extraction method by providing 76.89% rutin and 44.53% apigenin recoveries. As a result of this study, the maximum extraction conditions for rutin and apigenin were obtained as 160 bar, 40 °C, 0.35 mm particle size, 3 l/min CO_2, 8% w/w co-solvent ratio, and 120 min extraction period.

Keywords Rutin · Apigenin · Supercritical extraction · Soxhlet · *Berberis vulgaris* · Antioxidant

Introduction

The diversity of biological resources containing antioxidative polyphenol compounds represents a huge alternative to be used in adjunctive therapy. Nowadays, due to the disadvantages of synthetic drugs, the use of natural bioactive ingredients and extracts that do not contain toxic residues is increasing significantly. Some biologic extracts or pure natural compounds have been shown to be very active and therapeutic for serious pathological diseases in which only strong synthesized medicines have been used up to now. This potential and tendency could significantly reduce the dependence on chemically synthesized medicines. Concerning the therapeutic use of natural compounds, the wild medicine plant *Ranunculus macrophyllus Desf.* extracts were found to be quite successful in the treatment of acute skin inflammation and some skin disorders (Deghima et al. 2021). Isolated natural limonoids from the fruits of *Fortunella crassifolia* and the bark of *Citrus junos* were used in the treatment of human cervical cancer with Adriamycin (ADR) and were found to significantly increase the number of (HeLa) cancer cells dying (Kitagawa et al. 2021). *Berberis vulgaris* can be a new medical plant belonging to the *Berberidaceae* family. In a previous study, it was found that

Highlights

- *Berberis vulgaris* extracts have plenty of rutin. It is emphasized that rutin can be used against corona virus by inhibiting the main protease on the surface of COVID-19.
- Rutin and apigenin are very precious antioxidants.
- Soxhlet and supercritical carbon dioxide (Sc-CO_2) extraction methods were applied and compared as recovery.
- The effects of the parameters in the extraction process on the yield were evaluated and presented visually.

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the extracts taken from the *Berberis vulgaris* root bark by ultrasound-assisted extraction method have antioxidant, anti-melanogenic, and anti-inflammatory properties (Dulic et al. 2019). In another study, how the genetic types of *Berberis vulgaris* according to region characteristics in terms of their properties such as antioxidant, total phenolic, and flavonoid contents change was investigated (Okatan and Colak 2019).

Rutin and apigenin are popular antioxidants, and the research related to both are increasing day by day. The molecular structures of rutin and apigenin were given in Fig. 1. In a computer simulation study, rutin was found to have the highest inhibition effect among 51 different phytochemicals placed against the main protease of the COVID-19 virus. According to this study, it was observed that rutin is more effective against the COVID-19 virus than a commercial drug known as "lopinavir" (Al-Zahrani 2020). In another research, *Sorbus domestica* leaves were extracted using 70% methanol, and the maximum amount of rutin in the extract was determined as 2.1 mg/g (Rutkowska et al. 2019). Rutin, apigenin, and other polyphenols were extracted from *Lupin* seeds via Sc-CO_2 method to obtain extracts that are rich in polyphenols, and the amount of apigenin was found as 47.074 μg/g *Lupin* seed (Buszewski et al. 2019). Apigenin displays a high capacity of antioxidant and antiradical activity and can influence the activity of glutathione reductase and superoxide dismutase and important antioxidant enzymes (Espin et al. 2000). The antiradical ability of apigenin can be related to its chemical structure and particularly to positions of hydroxyl groups in the apigenin molecule (positions of hydroxyl group are 4, 5, and 7) (Benzie and Strain 1996). The antimicrobial effects of apigenin, tyrosol, hydroxytyrosol, oleuropein, and luteolin in olive were studied in two types of yeast, *Aureobasidium pullulans* and *Saccharomyces cerevisiae* (Canal et al. 2019). Similarly, in another study, extracts were prepared by the accelerated solvent extraction method using 70% methanol from *Cardus* species and nine flavonoids, including apigenin and phenolic acids, were detected in the extracts (Kozyra et al. 2019).

Sc-fluid extraction method is an advantageous and eco-friendly technology for pharmaceutical, cosmetic, and food applications, which are high value-added industries. Sc-CO_2 extraction method is applicable at low temperature and provides density-based selectivity, and this technique has effective diffusivity. Sc-CO_2 extraction method does not leave solvent residues. In addition, to remove CO_2 solvent, it is enough to simply decompress the pressure afterward supercritical fluid extraction (SFE) application. This method rarely needs an evaporation step. However, SFE also has certain disadvantages. Due to its structure, carbon dioxide has a low polarity, and when CO_2 is used alone as solvent, low yield occurs because of low solubility of polar substances. In the extraction of some active substances, the efficiency of the Sc-CO_2 method may be lower than traditional methods. In the literature, a high-pressure carbon dioxide extraction study was also conducted. It was reported that the effect of two parameters as pressure and extraction time was examined and the maximum anthocyanin, vitamin C, and TFC amounts were found to be 178.658 mg/l, 3468.7 mg/l, and 3298.15 mg GAE/l, respectively in the *Berberis vulgaris* fruits (Sharifi et al. 2019). In the literature, a species close to *Berberis vulgaris*, *Berberis crataegina* DC leaves, and fruits were extracted by ultrasonic assisted method, and the amounts of rutin and apigenin 7-O-glucoside were found as 170.87 μg/g 20.08 μg/g, respectively (Gulsoy et al. 2011). In another study, juices of Berberry type fruits were extracted with methanol/water solvent, and 7.61 mg/l rutin and 4.44 mg/l apigenin were identified by HPLC analysis (Gholizadeh-Moghadam et al. 2019).

To the best of our knowledge, this study is the first study investigating rutin and apigenin-rich oil of *Berberis vulgaris* fruits extracted by the Sc-CO_2 method with and without co-solvent, as well as the traditional Soxhlet method. Chemical characterization of these specific and precious flavonoids, apigenin and rutin, was determined by using HPLC with 4 channel UV (ultraviolet) detector.

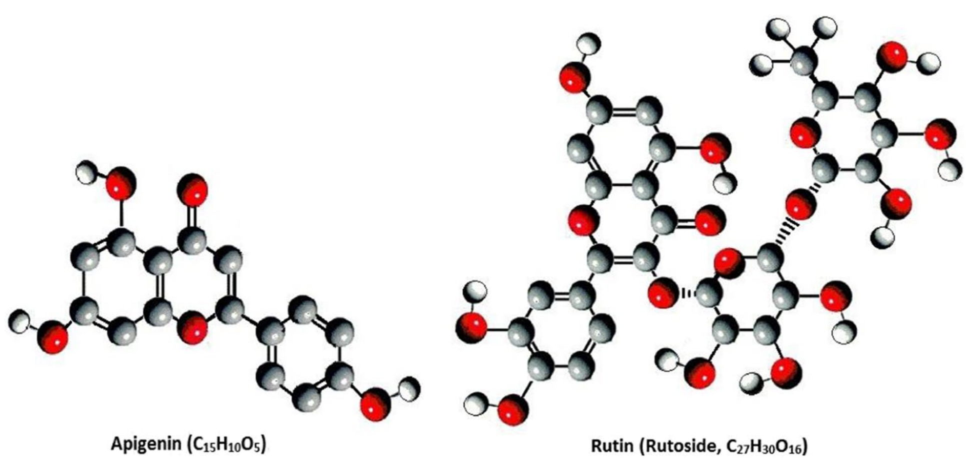


Fig. 1 Representation of apigenin and rutin. Atoms are represented with conventional color coding: hydrogen (white), carbon (black), and oxygen (red) (original content)

In the current Sc-CO_2 extraction study, the effects of six independent parameters (pressure, temperature, particle size, CO_2 flow rate, extraction period, and co-solvent percentage) were examined to identify optimum conditions of maximum rutin and apigenin amounts by using the parametric optimization method, and our results were presented with "% recovery" values according to the conventional Soxhlet method values.

Materials and Methods

Plant Material

Fresh and ripe *Berberis vulgaris* fruits were picked up as a raw material from Tarsus region of Mersin, Turkey. *Berberis vulgaris* samples were dried for 3 days in the shadow, downscaled by a laboratory crusher, and grinded into small size particles by Retsch (Haan, Germany) brand plant grinder and Bosch (Midrand, South Africa) brand coffee grinder, respectively. The moisture content of the identical samples was found 7.2% ± 0.1 w/w after drying in an oven at 105 °C for 120 min. All results in this study were given "µg substance per gram dry matter." The powder samples were classified into four different sizes by sieve analysis as mean 0.35 mm, 0.55 mm, 0.85 mm, and 1.00 mm.

Reference Standards and Chemicals

As a reference standard, rutin trihydrate (≥ 95%, referred to as rutin) and apigenin (≥ 95%) from Sigma Aldrich (Darmstadt, Germany) were utilized as reference standard materials. Methanol (purity ≥ 99.9%), acetonitrile (purity ≥ 99.9%), and ethanol (purity ≥ 99.9%) were used as mobile phase and as solvent to clean the extract collecting vessel. Solvents were purchased from Sigma-Aldrich (Darmstadt, Germany); formic acid (purity ≥ 99.9%) was supplied from Merck (Gernsheim, Germany) as a buffer for mobile phase. These chemicals were utilized for Soxhlet extractions, preparation of reference standards, and as co-solvent in Sc-CO_2 extractions and HPLC analyses. Siphoned liquid carbon dioxide (99.99%) was supplied as supercritical extraction solvent from Oksan gas company, Ankara, Turkey. Deionized water was supplied using Siemens SG brand reverse osmosis water purifier system (Munich, Germany).

Soxhlet Extraction

Soxhlet extraction is a traditional and reference method for comparing other extraction methods. Two powder samples of 10 g each of *Berberis vulgaris* fruits were extracted by Soxhlet method. Samples were taken at 0.35 mm average particle size and weighed in the Soxhlet cartridge. Extractions were applied for 7 h by using 170 ml ethanol as organic solvent in the Soxhlet apparatus. In the literature, similar conditions for this kind Soxhlet applications were used (Ghoreishi et al. 2016b). Ethanolic extracts were measured as 146 and 152 ml. The extracts were analyzed by the HPLC system for determining the amount of rutin and apigenin.

Supercritical CO_2 Extraction

In Sc-CO_2 extractions, solvent density, and solubility change with pressure–temperature relationship, the solving power of a Sc fluid increases with density at a given temperature, and the solving power of a Sc fluid at a given density increases with temperature. As the particle size decreases, the solving power increases due to the contact surface increasing. In addition of co-solvent, Sc conditions and polarity change according to the co-solvent properties and mole fractions, which can greatly increase the extraction efficiency (Arumugham et al. 2021). Laboratory scale SFE equipment, which is Spe-ed SFE model Applied Separation brand equipment, was used for the extraction of *Berberis vulgaris* samples throughout this study. The device consists of an air-driven liquid CO_2 pump, an air compressor, cooling and recirculating bath to cool air-driven liquid CO_2 pump, a high-pressure liquid pump for co-solvent pumping, an oven, an extractor chamber, an extractor, a CO_2 exit heater unit, a needle valve to arrange output flow rate of CO_2, a flowmeter to monitor CO_2 flow rate, and an extract collecting vessel. Liquid CO_2 from siphoned CO_2 tube is continuously sent through the cooled pressurizing pump of system, and in this unit, CO_2 mixing with co-solvent is performed under specified proportions. Then, the liquid pure CO_2 or modified with ethanol flows upwards through the extractor. Extractor has 24 ml internal volume. In this study, for the Sc-CO_2 extraction process, the extractor was prepared by placing glass wool under and over the sample of *Berberis vulgaris* (5 g of fruits per run). After extraction and depressurizing of solvent with outlet heater, liquid extracts were collected into the extract collecting vessels. Two quantitative responses were researched as rutin and apigenin in the extracts throughout this essay.

HPLC Analysis

The analysis of rutin and apigenin was performed with HPLC–UV system. Dionex 680 HPLC system (California, USA) with UVD 170U detector, P680 quaternary pump and an HPLC column was used to calculate the rutin and apigenin amounts in all extracts. HPLC column was an inertsil ODS 3 type 5 µm particle size 4.6 × 250 mm C-18 RP from GL-Science (Eindhoven, Netherlands). The elution solvents were formulated as follows: deionized water/acetonitrile/

formic acid at % 79.9:20:0.1, v/v (A) and 100% acetonitrile, v/v (B). The gradient HPLC program was performed with a constant flow rate of 1.0 ml/min, as follows: 0–1 min of 100% of A, 1–18 min 50–50% of A in B, 18–22.5 min 50–50% of A in B, 22.5–24 min 100% of A, and 24–25 min 100% A. The loop size of manual injection port of pump was 20 µl. The detector unit has four channel UV detection system. All absorption values were taken at 256 nm, and this wavelength was chosen suitable for both rutin and apigenin UV absorption spectrums. Stock standard solutions of rutin and apigenin were prepared in ethanol as 100 ppm and diluted for calibration curve to 5, 12.5, and 25 ppm as 3-point calibration standards.

Statistical Analysis

The study results were expressed as mean ± SD (standard error) of the replicated two measurements. The significant statistical difference throughout the measurements was determined using two tailed unpaired t-test. Statistical significance is considered at $p \leq 0.05$ significance level.

Results and Discussion

The independent parameters of Sc-CO_2 extraction for *Berberis vulgaris* were selected as pressure, temperature, mean particle size, CO_2 flow rate, co-solvent ratio, and extraction period. The parametric optimization was preferred in order to express the experimental effects of these six parameters more clearly. The each Soxhlet extraction was carried out for 7 h with 170 ml of ethanol. Ethanolic Soxhlet extracts were measured as 146 and 152 ml. The Sc-CO_2 extractions were performed at 30–150 min extraction period, 35–70 °C temperature, 140–240 bar pressure, 3–7 L /min CO_2 flow, 0.35–1.00 mm mean particle size, and 0–8% w/w co-solvent ratio. In this study, a limited proportion of polar co-solvent was added to Sc-CO_2 for increasing the total polarity and the bioactive component yield. There are studies at similar co-solvent addition ratios and parameter ranges to produce extracts containing higher concentrations of polyphenolic compounds. In a similar Sc-CO_2 extraction study to obtain apigenin and fisetin from Lupinus luteus seeds, the optimum conditions were found to be 73 °C, 147 bar, and 16% co-solvent ratio (Buszewski et al. 2019). In another Sc-CO_2 extraction study to obtain glycyrrhizin acid from *Glycyrrhiza glabra* (licorice) root, the optimum conditions were found as 68 °C, 296 bar, and 108 min extraction time (Hedayati and Ghoreishi 2015). Co-solvent flows were identified according to the flow rate of CO_2 by mass, and they were studied as 2.0%, 5.0%, and 8.0% w/w ethanol ratio. The influence of extraction parameters on the content of rutin and apigenin was investigated. All the extracts were stored at about − 6° C in a freezer to protect all flavonoid content until HPLC analysis.

These Sc-CO_2 extractions were limited to 8% w/w ethanol in order to maintain supercritical conditions and avoid high amounts of ethanol in extracts. These ethanolic extracts of Sc-CO_2 method and extracts of Soxhlet method were directly analyzed in the HPLC after micro filtration. In the HPLC analysis, R^2 values of calibration curves for rutin and apigenin were calculated as r^2:0.9998 and r^2:0.9955, respectively.

Extraction yields of rutin and apigenin were calculated with the following formulas as recovery.

$$RecoveryofApigenin = \frac{\frac{\mu gApigenin(byScCO_2)}{gRawmaterial}}{\frac{\mu gofextractableApigenin(bySoxhlet)}{gRawmaterial}} * 100 \quad (1)$$

$$RecoveryofRutin = \frac{\frac{\mu gRutin(byScCO_2)}{gRawmaterial}}{\frac{\mu gofextractableRutin(bySoxhlet)}{gRawmaterial}} * 100 \quad (2)$$

The final moisture content of the powder samples was measured as 7.2% ± 0.1 w/w, and all results are given per "g" dry matter. The identification and quantification of rutin and apigenin were performed by comparing with the retention times of characteristic chromatograms of reference standards and samples, and the quantification was calculated according to the peak areas. The chromatograms of reference standard and some samples were given in Figs. 2, 3, and 4.

Extraction Period Determination by Modeling

These studies were performed using experimental data showing the change of apigenin amount versus time. Initial working conditions were applied as 140 bar, 35 °C, 0.55 mm particle size, and 5 l/min CO_2. The extraction period was very important for high recovery and energy saving. The experimental results and modeling solutions are given in Fig. 5.

The apigenin content of *Berberis vulgaris* fruits samples was studied experimentally through time. In the light of the obtained experimental values, the possible maximum amount of apigenin was calculated when time approaches infinity. Time constant Tau (τ) was calculated for 95% confidence level by using Eqs. (3) and (4).

$$\frac{dA}{dt} = -\frac{1}{\tau}A \quad (3)$$

First order differential equation with initial condition $A_{(0)} = 0$.

Solution and general model:

$$A(t) = A_{t\to\infty}(1 - e^{-t/\tau}) \quad (4)$$

Coefficients (with 95% confidence bounds):

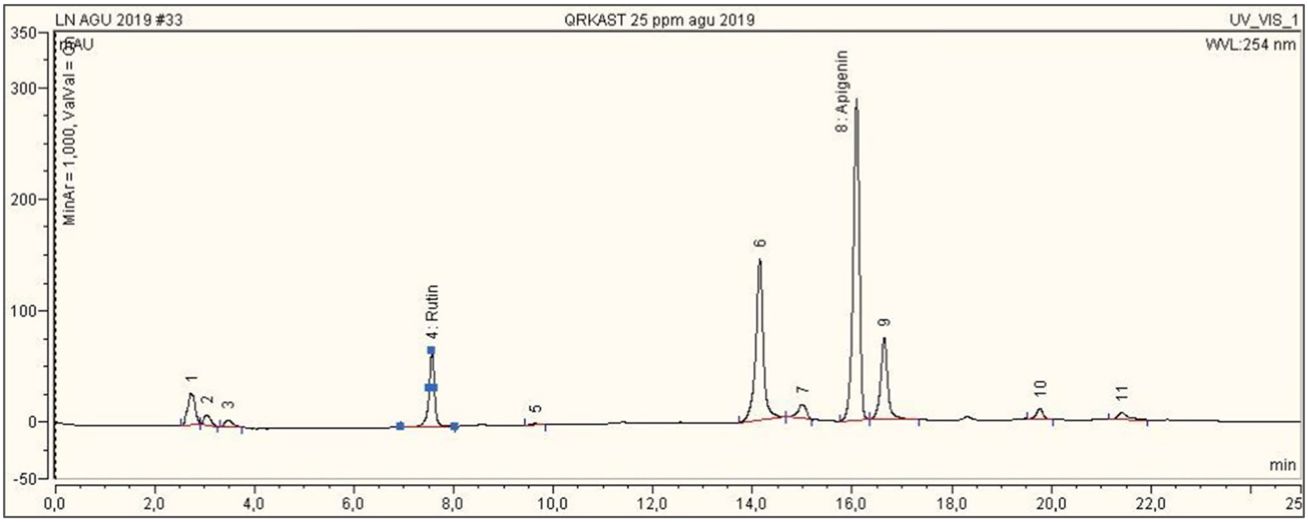


Fig. 2 The chromatogram of 25 ppm solution of rutin and apigenin reference standards at 254 nm

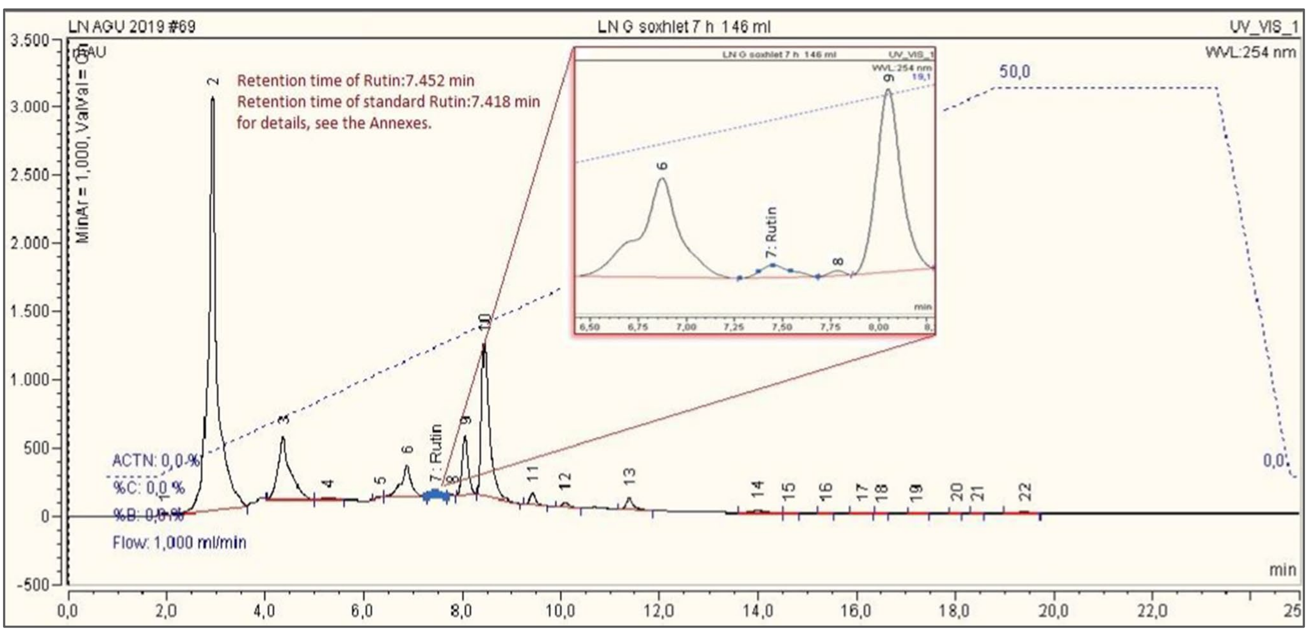


Fig. 3 The chromatogram of Soxhlet extract sample and the peak of rutin at 254 nm

$A_{t\rightarrow\infty}$ = 3.21 µg/g *Berberis vulgaris*, estimated maximum apigenin amount, when time approaches infinity.

τ_{1} = 0.8114 h (extraction time was calculated for $\tau_{2.46}$ (2 h) for 92% extraction efficiency).

R^2: 0.9912 (coefficient of variation) and RMSE: 0.0319 (root mean square error).

In the similar academic studies, the periods of Sc-CO_2 extractions were studied at various times in the range of 20–480 min. For astaxanthin and lutein extraction from *H. pluvialis*, the optimum extraction periods were found as 20 min and 40 min, respectively. (Molino et al. 2018). The bioactive substance extraction study from the leaves of *Duguetia furfuracea* was carried out for 180 min (Favareto et al. 2019). In the present study, extraction time was applied as 120 min throughout all experiments.

As can be seen in Fig. 5, nonlinear regression values of apigenin were very close to experimental R^2 values, and both values were close to 1. The regression value of time is 120 min to obtain 92% extraction yield of apigenin.

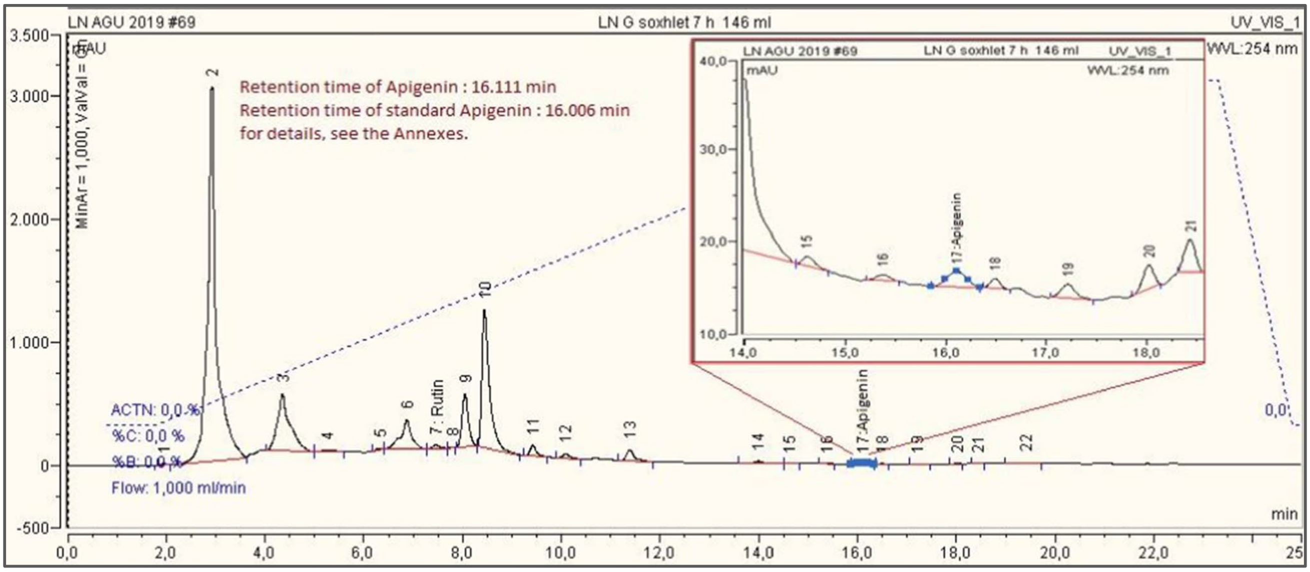


Fig. 4 The chromatogram of Soxhlet extract sample and the peak of apigenin at 254 nm

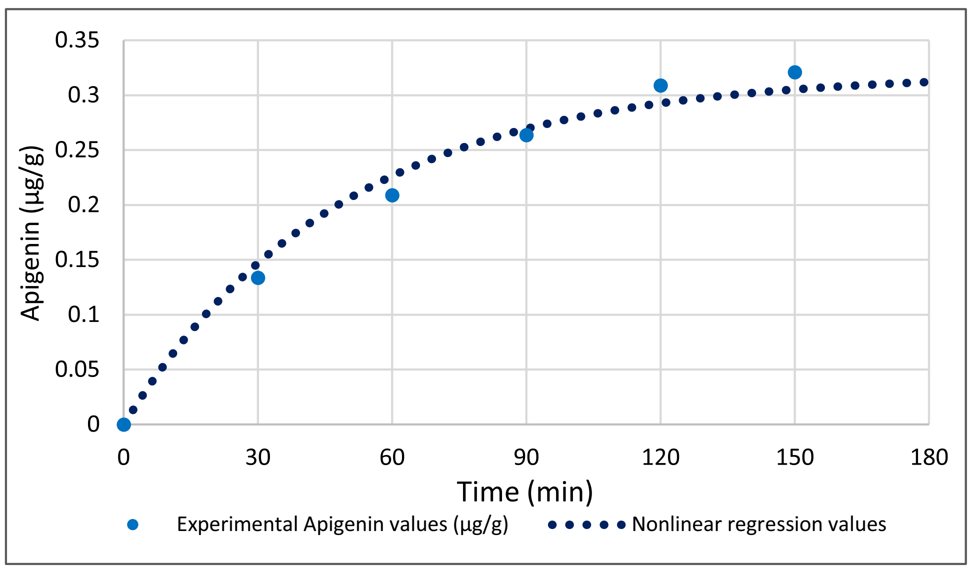


Fig. 5 Experimental and nonlinear regression values of apigenin amounts through time

Effect of Pressure on Extraction Recoveries

The pressure effect is generally the most important parameter in Sc-CO_2 studies. Pressure range was investigated in six stages, between 140 and 240 bar. Initial working conditions were applied as 35 °C, 0.55 mm particle size, 5 l/min CO_2, and previously determined 120-min extraction period. The experimental results are shown in Fig. 6.

The maximum rutin and apigenin amounts were found as 17.15 ± 0.31 µg/g and 0.381 ± 0.03 µg/g, respectively. The recovery values were calculated using Eqs. 1 and 2 as 7.62% and 5.82%, respectively. The amount of rutin and apigenin increased with increasing the operating pressure from 140 to 160 bar due to the dominant positive effect of increasing Sc-CO_2 density and solvation power. However, as the pressure increased from 160 to 240 bar, the amount of rutin and apigenin decreased, and since the convective mass transfer coefficient and permeability decreased, also the diffusion decreased. In a similar study based on the extraction of taxifolin from Pinus nigra bark, recovery increased as the pressure increased from 100 to 193 bar, but when the pressure was gradually increased from 193 to 300 bar, recovery values significantly decreased (Ghoreishi et al. 2016a).

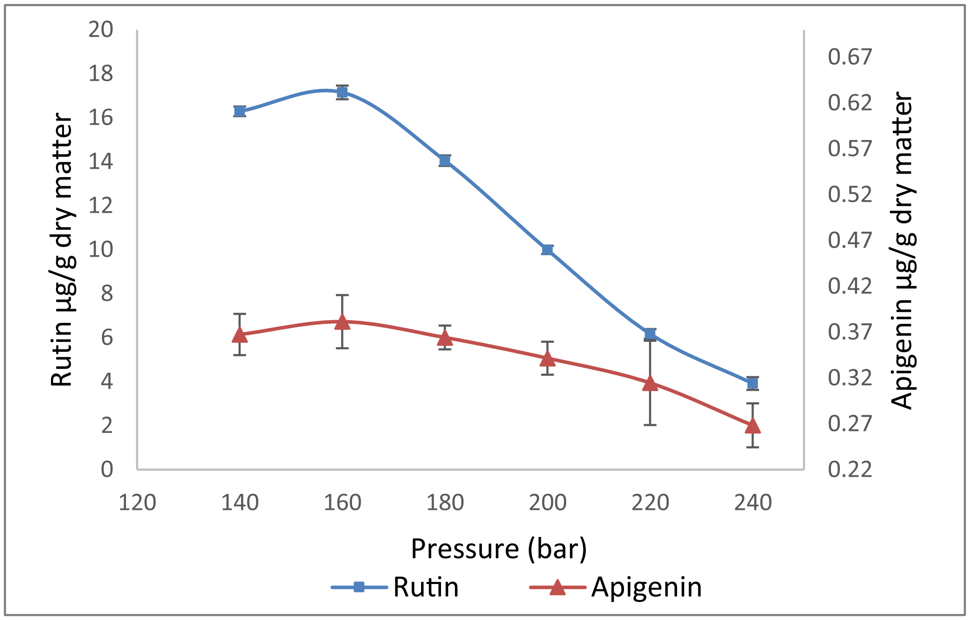


Fig. 6 The effect of pressure on the quantities of rutin and apigenin

Effect of Temperature on Sc-CO_2 Extraction Recoveries

In the Sc-CO_2 extractions, the effects of temperature were studied for the optimization of the amount of rutin and apigenin. Temperature range was selected and applied between 35 and 70 °C. Initial working conditions were fixed at 0.55 mm particle size, 5 l/min CO_2 flow rate, previously optimized 120-min extraction period, and 160 bar pressure. The experimental results were given in Fig. 7.

The maximum amount of rutin and apigenin were found as 17.15 ± 0.31 µg/g and 0.417 ± 0.017 µg/g, respectively. According to the Soxhlet data, maximum recoveries of rutin and apigenin were obtained as 7.62% and 6.38%, respectively. As can be seen in Fig. 7, while the temperature increased from 35 to 70, the amount of obtained rutin and apigenin from the extraction decreased by approximately 5 times. Since our study was based on apigenin optimization, extractions were continued at 40 °C, where the amount of apigenin is maximum.

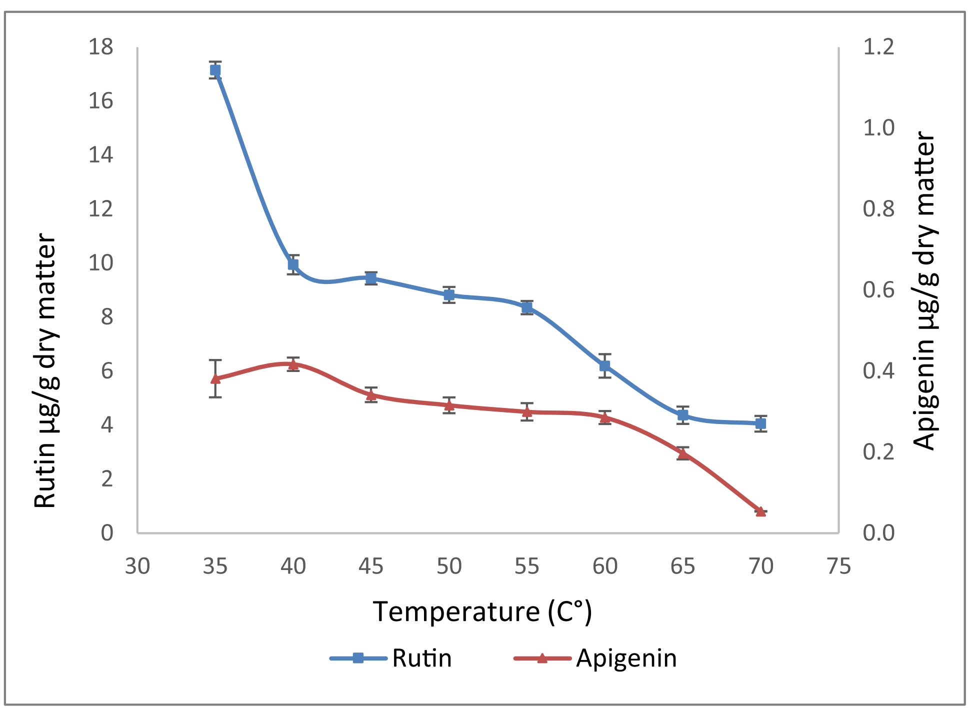


Fig. 7 The effect of temperature on the quantities of rutin and apigenin

When the extraction temperature raises from 35 to 70 °C, the Sc-CO_2 density decreases and its solvation power also decreases, such as gases. There are basically two factors affecting the recovery: vapor pressure and solubility of bioactive components. These two factors affect the increase or decrease of obtained rutin and apigenin amounts. In this study, the dominant factor was observed as decreasing of Sc-CO_2 density and its solvating power. The similar results of some researchers were reported about extraction yields from different sources in the literature. The extraction yield of nutmeg oil decreased when the temperature increased from 313 to 323 K (Machmudah et al. 2006). In a milk thistle Sc-CO_2 extraction study, the amount of silybin and oil decreased with increasing temperature (Çelik and Gürü 2015).

Effect of Particle Size on Extraction Recoveries

The particle size effect on the Sc-CO_2 extraction recovery was investigated for the optimization of the amount of rutin and apigenin. Extractions were carried out using samples with an average particle size of 0.35, 0.55, 0.85, and 1.00 mm. Working conditions were fixed at previously optimized 160 bar pressure, 40 °C, 120 min extraction period, and 5 l/min CO_2 flow rate. The extraction results are given in Fig. 8.

At this step, the results of maximum amounts of rutin and apigenin from *Berberis vulgaris* were found as 10.56 ± 0.35 μg/g and 0.52 ± 0.026 μg/g, respectively. The recoveries of rutin and apigenin were obtained as 4.69% and 7.95%, respectively.

As can be seen in Fig. 8, when the particle size of the sample was decreased from 1.00 to 0.35 mm, the amount of rutin and apigenin increased by 74.1% and 378%, respectively. When the smaller particle sizes are used, the diffusion pathway of the solvent Sc-CO_2 in the solid phase is shortened, and internal mass transfer resistances of solid decrease; therefore, the extraction efficiency increases due to much more Sc-CO_2 penetrating into the sample. In a study conducted on *Elaeagnus* seeds, it was observed that the amount of quercetin in extracts taken by Sc-CO_2 extraction increased by 73.3% as a result of decreasing the particle size (Nuralin et al. 2017). Maximum recoveries of both rutin and apigenin were obtained with 0.35 mm particle size samples and selected as the optimal particle size value in the following extractions.

Effect of CO_2 Flow Rate on Extraction Recoveries

In this study, the CO_2 flow rate effect was analyzed, to obtain maximum amount of rutin and apigenin in the range of 3–7 l/min CO_2 and investigated at 5 different flow rates. Previously determined optimum conditions for pressure, temperature, extraction time, and particle size were found as 160 bar, 40 °C, and 120 min and 0.35 mm, respectively. The experimental results were given in Fig. 9.

The maximum amount of rutin and apigenin were found as 11.13 ± 0.42 μg/g and 0.616 ± 0.03 μg/g, respectively. The recoveries of rutin and apigenin were found as 4.94% and 9.41%, respectively, at 3 l/min CO_2 flow rate. As can be seen in Fig. 9, when the behavior of extraction efficiency against the Sc-CO_2 flow rate was examined, it was seen that the

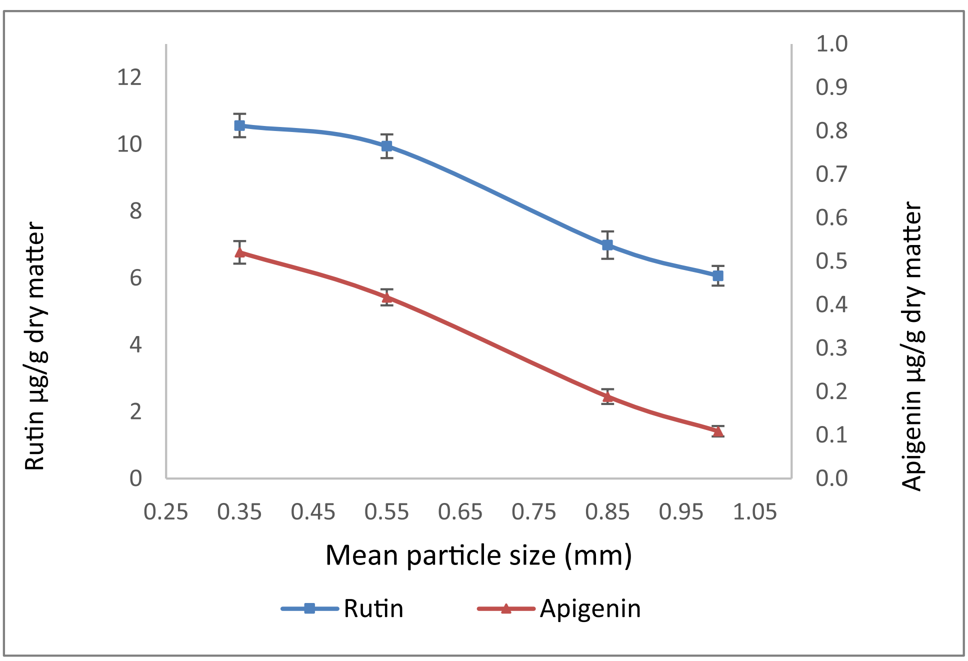


Fig. 8 The effect of particle size on the quantities of rutin and apigenin

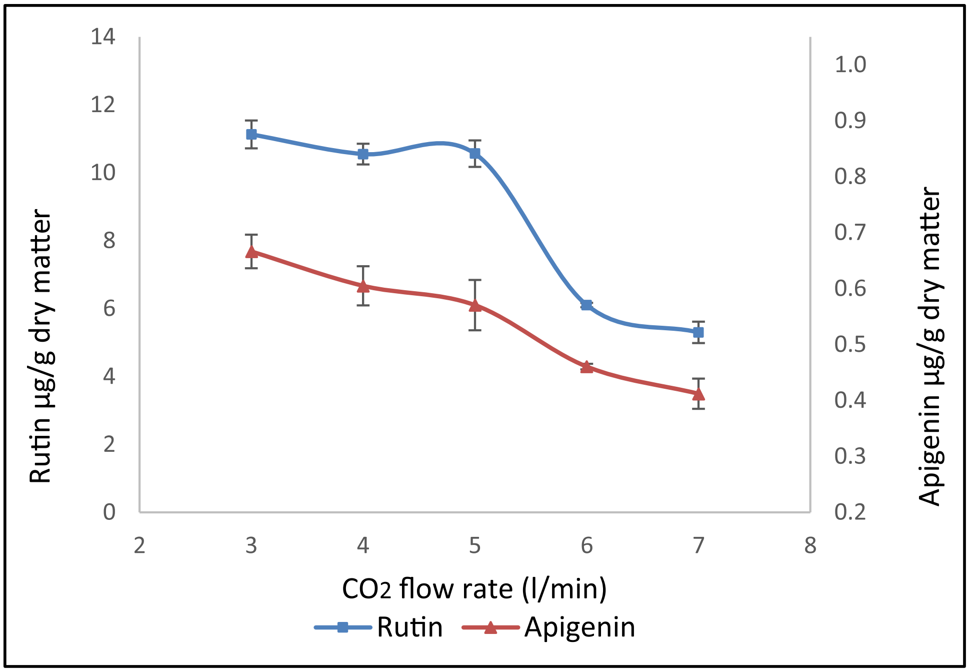


Fig. 9 The effect of CO_2 flow rate on the quantities of rutin and apigenin

amount of rutin and apigenin in the extract decreases as the flow rate increases. Increasing the CO_2 flow rate increases the linear flow rate in the extractor, thereby reducing the contact time between the sample and the solvent. Short-term contact causes lower extraction recovery In a literature, oil and phytosterol extraction from the Kalahari melon seeds, It was found that the amount of oil increased at the beginning but then decreased and gave maximum value at the CO_2 flow rate of 15 ml/min. However, as the CO_2 flow rate increased, the amount of phytosterols decreased continuously (Nyam et al. 2011).

Effect of Co-solvent Adding on Extraction Recoveries

According to the results obtained in the previous parametric optimization studies, the obtaining conditions of the maximum rutin and apigenin amounts were determined. These conditions can be seen in Figs. 5, 6, 7, 8, and 9 as 160 bar, 40 °C, 0.35 mm mean particle size, 3 l/min CO_2, and 120 min. Ethanol has a little toxicity effect and high polarity; it was preferred as co-solvent. Ethanol was fed as 0.14, 0.34, and 0.55 ml/min flows to provide 2%, 5%, and 8% by mass to the extractor besides liquid CO_2. Total volumes of ethanolic extracts were measured as 8, 12.5, and 32 ml, respectively. The concentration calculations of rutin and apigenin were performed by using these volume factors. The critical temperature of ethanol is much higher than carbon dioxide's. Therefore, the critical temperature of the ethanol-CO_2 mixture will be higher than that of pure carbon dioxide. With the addition of 2%, 5%, and 8% w/w ethanol, the mole fractions of carbon dioxide were approximately 0.98, 0.952, and 0.923; the critical temperatures in these conditions were calculated by interpolation and found as 36.24 °C, 42.47 °C, and 47.11 °C, respectively (Chatwell et al. 2021). When optimum conditions were applied with these ethanol-CO_2 ratios, 5% and 8% co-solvent added studies were carried out under subcritical conditions since they were performed at constant 40 °C. These experiments were limited to 8% ethanol addition due to the formation of a high volume of ethanolic extract and the cost effect of the ethanol evaporation step. These ethanolic extracts were analyzed directly by the HPLC after micro filtration.

As shown in Fig. 10, in the subcritical-CO_2/Sc-CO_2 extractions, the amount of rutin increased from 17.15 ± 0.31 to 173 ± 14.97 µg/g, and apigenin increased from 0.616 ± 0.03 to 2.92 ± 0.11 µg/g with the addition of 8% ethanol as co-solvent. When the ethanol addition rate was increased from 0 to 8% w/w, the polarity of the mix solvent increased, so the obtaining amounts of rutin and apigenin increased significantly. It should be noted that the "Soxhlet with 100% ethanol" data in Fig. 10 is the only conventional reference extraction method data and is used only to calculate recoveries and to compare Soxhlet and subcritical-CO_2/Sc-CO_2 extraction method efficiencies.

Comparison of Operation Conditions of Methods in Terms of Recoveries

Operation conditions of all the applied extraction methods and their maximum rutin and apigenin amounts and recoveries are given in Table 1.

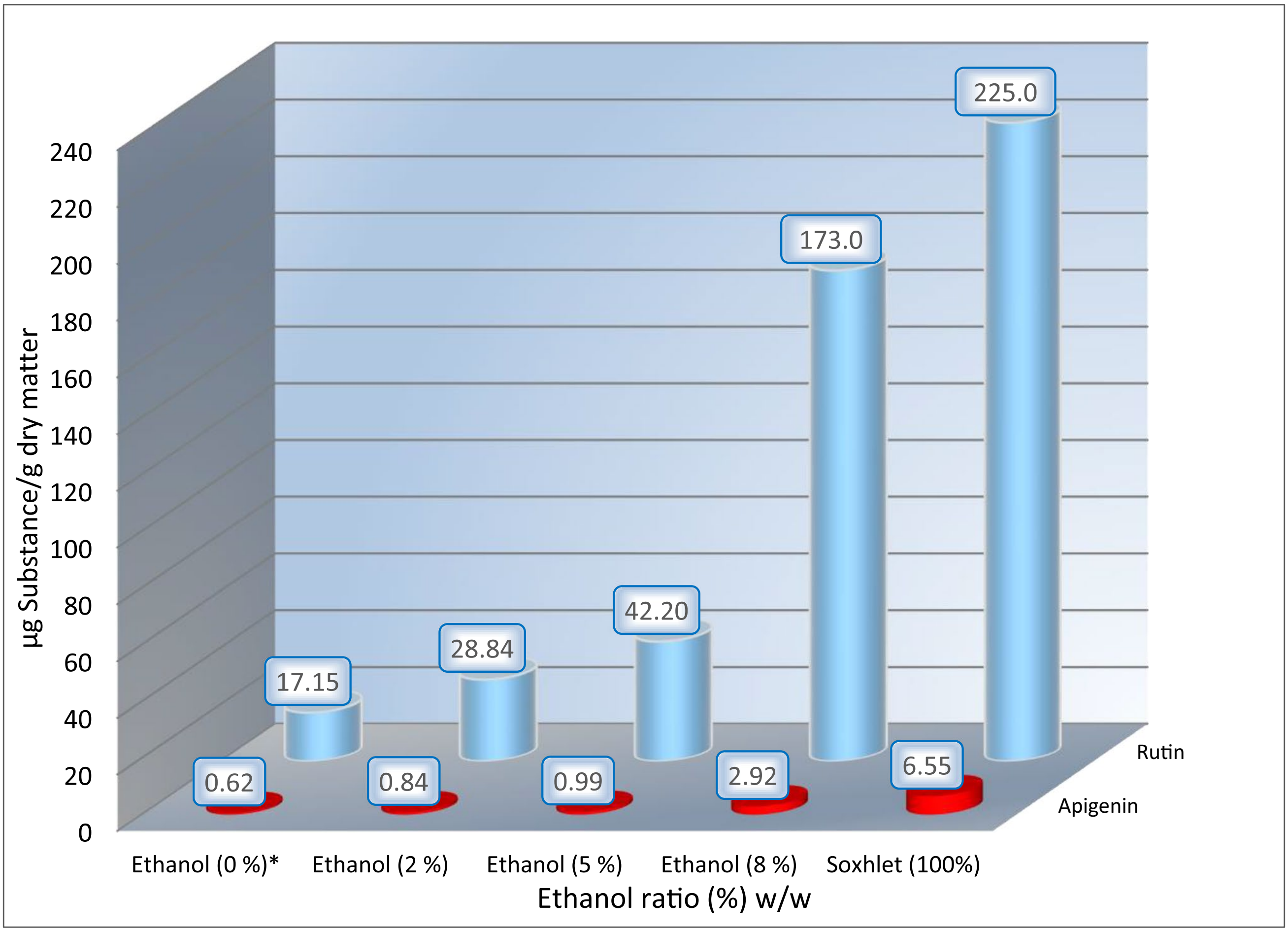


Fig. 10 The change of rutin and apigenin with different ethanol percentages w/w as co-solvent. Single asterisk indicates that the results of rutin and apigenin were obtained via Sc-CO2 extraction without co-solvent

Table 1 The optimal conditions of methods, rutin and apigenin amounts, and recoveries

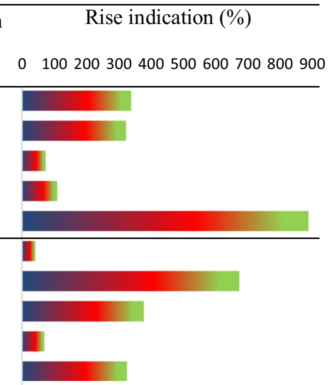
Extraction method	Optimal operation conditions	Extraction time (min)	Total volume (ml)	Rutin/apigenin (µg/g)	Recovery rutin/apigenin (%)
Soxhlet extraction	With 170 ml ethanol	420	146	225.01/6.55	100/100
Sc-CO_2 optimization without co-solvent	160 bar, 40 °C, 0.35 mm, 3 l/min CO_2	120	0.6	17.15/0.616	7.62/9.4
Subcritical-CO_2 optimization with 8.0% w/w EtOH	160 bar, 40 °C, 0.35 mm, 3 l/min CO_2	120	32	173/2.92	76.88/44.58

This study is the first experimental investigation for the parameter optimization of *Berberis vulgaris* fruit to identify the rutin and apigenin content via Soxhlet and Sc-CO_2 extraction methods. The results showed that the Soxhlet extraction provides a yield of 225.01 µg/g ± 13.45 rutin and 6.55 µg/g ± 0.21 apigenin. Parametric optimization conditions were obtained in Sc-CO_2 studies as 160 bar pressure, 40 °C, 0.35 mm mean particle size, 3 l/min CO_2 flow rate, 120-min period, and 8% w/w ethanol. Significant and insignificant independent parameters on extraction yields are presented in Table 2.

In the literature, generally berberine and palmatine alkaloids have been studied in *Berberis* species. In a study on *Berberis vulgaris*, 145.5 µg/ml berberine was obtained by ultrasound-assisted extraction with a water glycerol mixture (Dulic et al. 2019). In another study, after the microwave-assisted extraction process of *Berberis Jaeschkeana* and *Berberis Asiatica* species, the berberine and palmatin

Table 2 The effect diagram of independent parameters on the yields of rutin and apigenin

Substances soluted by Sc-CO_2	Indipendent parameters	Parameter Ranges	Risen (%)
Rutin	Pressure	From 240 to 160 bar	339
	Temperature	From 70 to 35 °C	323
	Particle size	From 1 to 0.35 mm	74
	CO_2 flow rate	From 7 to 3 l/min CO_2	110
	Co-solvent percentage	From 0 to 8 % w/w[1]	908
Apigenin	Pressure	From 240 to 160 bar	42
	Temperature	From 70 to 40 °C	674
	Particle size	From 1 to 0.35 mm	378
	CO_2 flow rate	From 7 to 3 l/min CO_2	70
	Co-solvent percentage	From 0 to 8 % w/w[1]	257



[1]The increments in the amount of rutin and apigenin were calculated and compared according to the Sc-CO_2 studies with and without 8% co-solvent as can be seen in Fig. 10.

contents were found as 46.4 mg g − 1 20.5 mg g − 1 for B. *Jaeschkeana* and 88.7 mg g − 1 18.7 mg g − 1 for B. *Asiatica*, respectively (Belwal et al. 2020).

As can be seen from Table 2, the parameters affecting the extraction efficiency were found as co-solvent ratio, temperature, and pressure, respectively.

Conclusions

For the first time in the literature, this paper aimed to achieve the highest rutin and apigenin extraction efficiency from *Berberis vulgaris* via using conventional extraction process and subcritical-CO_2/Sc-CO_2 extraction process by comparing the results of these methods with the parametric optimization. Moreover, comparison studies were also carried out to obtain recoveries of rutin and apigenin by Soxhlet extraction. In the present study, the effects of pressure (140–240 bar), temperature (35–70 C), particle size (0.35–1.0 mm), CO_2 flow rate (3–7 l/min CO_2) co-solvent ratio (0–8% w/w), and time (0.5–2.5 h) on extraction from *Berberis vulgaris* fruits via Sc-CO_2 with/without co-solvent were investigated. The findings of this study show that the Sc-CO_2 extraction method is almost as efficient as the Soxhlet method in extracting active phenolic content. When the obtained results by the subcritical-CO_2 were compared with the results of Soxhlet method, which is the conventional method, the recoveries of maximum rutin and apigenin were found as 76.88% and 44.58%, respectively. How the parameters affect the yield percentages of rutin and apigenin can be seen in Table 2.

The most striking independent parameter to increase the efficiency of rutin and apigenin was the addition of co-solvent. As a result of the addition of 8% ethanol as co-solvent, the amounts of rutin and apigenin rose up to 173 ± 14.97 µg/g and 2.92 ± 0.11 µg/g, respectively. There are literature indicating the importance of rutin in one of these studies showing that the rutin is more effective than Lopinavir, which is used as a medicine against COVID-19 virus (Al-Zahrani 2020). In this study, rutin amounts were determined as 225.01 ± 13.4 µg/g and 173 ± 14.9 µg/g by Soxhlet and subcritical-CO_2 extraction methods, respectively. These amounts are very promising results, showing that *Berberis vulgaris* fruits can be used as raw material of a medicine against COVID-19 virus.

Considering the potential of the *Berberis vulgaris* plant, it would be appropriate to continue the research with subcritical water extraction and Sc-CO_2 extractions with glycerine/ethanol mixture as a new co-solvent. These comprehensive parametric optimization results could be utilized by pharmaceutical industry for preparing new drugs or food industry for preparing new food supplements. Process engineers working on scaling up in subcritical and Sc fluid extractions can also use these results for flavonoid or antioxidant extractions. Besides, researchers can use these results to increase the efficiency of their own processes.

Abbreviations Sc-CO_2: supercritical carbon dioxide; Sc: supercritical; HPL: Chigh-pressure liquid chromatography; T1FC: total phenolic content; GAE: gallic acid equivalent; UV: ultraviolet; R^2: square of correlation coefficient; τ: time constant (Tau); RMSE: root mean square error

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Code Availability No special software was used in this study.

Declarations

Ethics Approval Live experimental objects were not used in this study. The study was conducted in accordance with ethical rules.

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