Berberis Vulgaris Fruit: Determination of Phenolic Compounds in Extracts Obtained by Supercritical CO₂ and Soxhlet Methods Using HPLC

Levent Nuralın1 · Metin Gürü1

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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-CO₂) 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 CO₂ flow rates, and 0–8% w/w co-solvent. As a result of Sc-CO₂ 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-CO₂ method was compared with the Soxhlet method, it was seen that the fast and eco-friendly Sc-CO₂ 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 CO₂, 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-CO₂) 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.

1 Department of Chemical Engineering, Engineering Faculty of Gazi University, Ankara, Turkey
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-CO₂ 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-CO₂ extraction method is applicable at low temperature and provides density-based selectivity, and this technique has effective diffusivity. Sc-CO₂ extraction method does not leave solvent residues. In addition, to remove CO₂ 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 CO₂ 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-CO₂ 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 and 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-CO₂ 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.
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, down-scaled 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 solvating power of a Sc fluid increases with density at a given temperature, and the solvating power of a Sc fluid at a given density increases with temperature. As the particle size decreases, the solvating 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 (Arunugham 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 ≤ 0.05 significance level.

**Results and Discussion**

The independent parameters of Sc-CO₂ extraction for *Berberis vulgaris* were selected as pressure, temperature, mean particle size, CO₂ 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-CO₂ extractions were performed at 30–150 min extraction period, 35–70 °C temperature, 140–240 bar pressure, 3–7 L/min CO₂ 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-CO₂ 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-CO₂ 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-CO₂ 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 CO₂ 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 −6 °C in a freezer to protect all flavonoid content until HPLC analysis.

These Sc-CO₂ 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-CO₂ 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.

\[
\text{Recovery of Apigenin} = \frac{\mu g_{	ext{Apigenin(by Sc CO}₂)} \text{g}_{\text{Raw material}}} {\mu g_{\text{ extractable Apigenin(by Soxhlet)} \text{g}_{\text{Raw material}}}} \times 100
\]

(1)

\[
\text{Recovery of Rutin} = \frac{\mu g_{\text{Rutin(by Sc CO}₂)} \text{g}_{\text{Raw material}}} {\mu g_{\text{ extractable Rutin(by Soxhlet)} \text{g}_{\text{Raw material}}}} \times 100
\]

(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 CO₂. 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 ($\tau$) was calculated for 95% confidence level by using Eqs. (3) and (4).

\[
\frac{dA}{dt} = -\frac{A}{\tau}
\]

(3)

First order differential equation with initial condition $A_{01} = 0$.

Solution and general model:

\[
A(t) = A_{\infty}(1 - e^{-t/\tau})
\]

(4)

Coefficients (with 95% confidence bounds):
\[ A_{t \rightarrow \infty} = 3.21 \, \mu g/g \] Berberis vulgaris, estimated maximum apigenin amount, when time approaches infinity.

\[ \tau_1 = 0.8114 \, h \] (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.
Effect of Pressure on Extraction Recoveries

The pressure effect is generally the most important parameter in Sc-CO₂ 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 CO₂, 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-CO₂ 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).

![The chromatogram of Soxhlet extract sample and the peak of apigenin at 254 nm](Fig. 4)

![Experimental and nonlinear regression values of apigenin amounts through time](Fig. 5)
Effect of Temperature on Sc-CO₂ Extraction Recoveries

In the Sc-CO₂ 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 CO₂ 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.
When the extraction temperature raises from 35 to 70 °C, the Sc-CO₂ 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-CO₂ 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-CO₂ 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-CO₂ 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 CO₂ 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-CO₂ in the solid phase is shortened, and internal mass transfer resistances of solid decrease; therefore, the extraction efficiency increases due to much more Sc-CO₂ penetrating into the sample. In a study conducted on *Elaeagnus* seeds, it was observed that the amount of quercetin in extracts taken by Sc-CO₂ 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 CO₂ Flow Rate on Extraction Recoveries**

In this study, the CO₂ flow rate effect was analyzed, to obtain maximum amount of rutin and apigenin in the range of 3–7 l/min CO₂ 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 CO₂ flow rate. As can be seen in Fig. 9, when the behavior of extraction efficiency against the Sc-CO₂ flow rate was examined, it was seen that the

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

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0.25 0.35 0.45 0.55 0.65 0.75 0.85 0.95 1.05

0.0 0.25 0.5 0.75 1.0

0.0 0.2 0.4 0.6 0.8 1.0

Mean particle size (mm)

Rutin µg/g dry matter

Apigenin µg/g dry matter

Rutin

Apigenin

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amount of rutin and apigenin in the extract decreases as the flow rate increases. Increasing the CO₂ 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 CO₂ flow rate of 15 ml/min. However, as the CO₂ 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 CO₂, 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 CO₂. 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-CO₂ 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-CO₂ 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-CO₂/Sc-CO₂ 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-CO₂/Sc-CO₂ 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.
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-CO₂ 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-CO₂ studies as 160 bar pressure, 40 °C, 0.35 mm mean particle size, 3 l/min CO₂ 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...
contents were found as 46.4 mg g⁻¹, 20.5 mg g⁻¹ for B. J aeschkeana 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-CO₂/Sc-CO₂ 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), CO₂ flow rate (3–7 l/min CO₂) co-solvent ratio (0–8% w/w), and time (0.5–2.5 h) on extraction from Berberis vulgaris fruits via Sc-CO₂ with/without co-solvent were investigated. The findings of this study show that the Sc-CO₂ extraction method is almost as efficient as the Soxhlet method in extracting active phenolic content. When the obtained results by the subcritical-CO₂ 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. 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-CO₂ 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-CO₂ 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-CO₂: supercritical carbon dioxide; Sc: subcritical
- HPL: High-pressure liquid chromatography; T1FC: total phenolic content; GAE: gallic acid equivalent; UV: ultraviolet; R²: square of correlation coefficient; τ: time constant (Tau); RMSE: root mean square error

## Supplementary Information

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## Author Contribution

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## Availability of Data and Material

The data of this study are accessible data.
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