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ALEKSANDRA BOGDANOVIC[1]
VANJA TADIC[2]
SLOBODAN PETROVIC[1]
DEJAN SKALA[1]

[1]University of Belgrade, Faculty of Technology and Metallurgy, Belgrade, Serbia
[2]Institute for Medical Plant Research "Dr Josif Pancic", Belgrade, Serbia

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# SUPERCRITICAL $CO_2$ EXTRACTION OF STEROIDAL SAPOGENINS FROM FENUGREEK (*Trigonella foenum-graecum* L.) SEED

## Article Highlights

- Supercritical extraction of steroidal sapogenins from fenugreek was examined
- The procedure of sapogenins extraction from fenugreek by supercritical extraction was described
- The influence of process parameters on sapogenins extraction was determined
- Optimization of steroidal sapogenins supercritical extraction from fenugreek was examined

## Abstract

*Supercritical $CO_2$ extraction was applied on fenugreek (Trigonella foenum-graecum L., Fabaceae) seeds with the aim to define optimal process conditions to obtain the maximal extract yields content of steroidal sapogenins. Central composite rotatable design (CCRD) combined with response surface methodology (RSM) was applied to determine optimal process conditions defined by the influence and interaction of pressure, temperature and time of extraction through consumption of SC $CO_2$. Optimization experiments revealed that the pressure of 24.73 MPa, the temperature of 38.2 °C and the consumption of SC $CO_2$ of 19.24 g/$g_{DM}$ were the best process conditions enabling the maximal yield of extract and gain of the highest content of sapogenins. The optimal values of pressure and temperature defined SC $CO_2$ density of 885.47 kg/$m^3$, which provided the maximal yield of the extract with the highest content of steroidal sapogenins. The achieved yield of extract at these conditions was 0.073g/$g_{DM}$, with 0.774 mg/$g_{DM}$ diosgenin, 0.477 mg/$g_{DM}$ protodioscin, 0.713 mg/$g_{DM}$ sarsapogenin and 0.205 mg/$g_{DM}$ oleanolic and ursolic acid with a significant quantity of 2.475 mg/$g_{DM}$ in the obtained extract.*

*Keywords: sapogenins, fenugreek, optimization.*

Fenugreek (*Trigonella foenum-graecum* L.) has been applied in traditional medicine for its beneficial health effects for centuries. Steroidal saponins, as bioactive phytochemicals of importance, occur abundantly in *T. foenum-graecum* [1-2]. The main representative, diosgenin, has been known for its use in the pharmaceutical industry as the main precursor of steroidal hormones [3-5]. In addition to diosgenin, other steroidal sapogenins, namely sarsapogenin and protodioscin, detected in fenugreek possessed significant anticancer, hypocholesterolemic, and antidepressant activities [6-12], exhibited the beneficial effects on the cardiovascular system [10], and might take a part in the regulation of glycemia and high blood pressure [8-9,13]. Besides, oleanolic and ursolic acids are known for different biological activities, their ability to reduce blood pressure, to regulate cholesterol level, and to induce the apoptosis of cancer cells [14-20]. Interestingly, ursolic acid represents one of the most important constituents of fenugreek seed. In the literature so far, it was reported to be present only in its ethanol-aqueous extract [16-22]. The concurrent presence of oleanolic acid and ursolic acid, its isomer, in plant material might be responsible for their

Correspondence: A. Bogdanovic, Department of Organic Chemical Technology, Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11000 Belgrade , Serbia.
E-mail: abogdanovic@tmf.bg.ac.rs
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possible synergistic effect on several health impairments [23-25]. As the importance and application of ursolic acid have been highlighted through numerous researches performed in recent years, one of the goals of our work was to define optimal extraction conditions that might enable obtaining the extracts rich in this metabolite.

Related to significant saponins bioactivity, biocompatibility, and wide application, highlighting their use in the synthesis of steroid drugs in pharmaceutical industries, as well as their application in the treatment of hypercholesterolemia, hyperglycemia, hypertension and cancer, the extraction of saponins from natural resources has been become a focus of interest. But, steroidal saponins were mostly investigated in the *Dioscorea* species, as an economically justified plant source for their extraction. Therefore, due to the increasing demand for these compounds, but limited plant resources, with the specific advantage of fenugreek being more easily cultivated and having a more rapid growth in comparison to *Dioscorea*, fenugreek seed, representing the abundant source of steroidal sapogenins, might be set as their profitable source in industry application [1,21,26-30].

Because of the wide pharmacological properties useful in the treatment of many health disorders, obtaining fenugreek seed extracts rich in saponins attracted the attention of the scientific community [3-4,7,9-12,17,20,22-23,26-27]. Supercritical fluid extraction (SFE) was applied as one of the most promising extraction techniques, with the advantages in comparison to traditional techniques, being that obtained supercritical extract is free of organic solvents, the SFE extraction is much faster without the necessity to employ the steps of further purification, making the final product much more economical. As presented in our previous work, in order to achieve the high efficiency, the supercritical (SC) extraction of diosgenin from fenugreek seeds required pre-treatment procedure of the plant material, comprising of defatting as the first step, followed by acid hydrolysis [27].

In this work, the wide range of working conditions of SFE was examined to ascertain the optimal process parameters of pressure, temperature and consumption of $CO_2$ (at different times of the process) which would enable extracts rich in diosgenin, sarsapogenin, protodioscin, ursolic and oleanolic acids. This study aimed to establish a comprehensive mathematical model, defining the influence of SC extraction process parameters and the best operating conditions. The optimal working condition of process extraction was determined, based on the applied wide range of pressure, temperature, and consumption of SC solvent, by response surface methodology (RSM) and central composite design (CCD). The detailed experimental plan was designed using the RSM and CCD, which gave the information related to the influence of process parameters on total steroidal sapogenins yields, their interactive effect and signification on the final most abundant content of these compounds in the obtained extracts. Considering the high potential of saponins as bioactive compounds and their content in fenugreek seeds, the present study was accomplished to examine fenugreek extract abundant in these secondary metabolites. Determining optimal condition parameters of applied SC extraction, the extracts rich in sapogenins were obtained. Defining the optimal conditions of SC extraction efficiency, through examined process parameters, might establish fenugreek as a commercially acceptable and significant source of steroidal saponins.

## EXPERIMENTAL

### Plant material

The seeds of *Trigonella foenum-graecum* L., Fabaceae (fenugreek) were collected in July 2014 in the province of Vojvodina, the northern part of Serbia. The voucher specimen was deposited at the Institute for Medicinal Plant Research “Dr Josif Pancic”, N$^o$ 11550412. Two hundred grams of seeds were air-dried and milled in a blender for 60 s every single time in advance to defatting and acid hydrolysis.

### Pretreatment of plant seeds

#### *Defatting*

Fenugreek seed with were milled in advance to defatting by refluxing with 625 mL hexane in a Soxhlet apparatus for 4 h. The hexane was evaporated to dryness. The yield of collected fat was in the range of 0.70±0.2%.

#### *Acid hydrolysis*

Hydrolyzation of defatted air-dried seeds was performed with 2 M HCl at 100 °C for 3 h (plant material:HCl = 1:4) in a vessel with a magnetic stirrer. After hydrolysis, the mixture was cooled and filtered under vacuum by Buchner apparatus. Neutralization of filtered sediment was performed by 5% $Na_2CO_3$ and distilled water, washed and filtered through the filter paper until neutral pH value was obtained. Neutralized and filtered sediment was dried in vacuum at 80 °C to a constant moisture content of less than 5%.

### Supercritical $CO_2$ extraction-SFE

Extraction was carried out in a semi-batch Autoclave Engineers Screening System previously described in detail [31]. The extractor was filled with approximately 40 g of dried defatted and hydrolyzed seeds of fenugreek. The extraction of prepared material at the selected process conditions of pressure, temperature, and different applied time was realized. The obtained extracts were collected into sample containers and the total extract yields were calculated. Extracts were kept in a refrigerator (at -5 °C) for the quantitative determination of diosgenin, sarsapogenin, protodioscin, ursolic and oleanolic acids, as steroidal sapogenins. Quantitative analysis of predicted compounds in obtained extracts was obtained by high-performance liquid chromatography (HPLC).

### Analysis of steroidal sapogenins

Quantification of steroidal sapogenins content was performed by HPLC (Agilent Technologies 1200). Detection was carried out using a diode array detector (DAD), and chromatographs were recorded at $\lambda$ = 210 nm. HPLC analysis of the investigated samples was achieved using a LiChrospher100 RP 18e, 250 mm×4 mm i.d. column, using isocratic elution with a flow rate of 1 mL/min. The mobile phase was gradient programmed from 5% of acetonitrile isocratic for 3 min, followed by 27 min linear elution of 95% of acetonitrile and 13 min isocratic elution of 95% acetonitrile. The column temperature was controlled at 30 °C. The samples were prepared by dissolving 50 mg extracts (obtained by the previously described procedure) in 1 mL of MeOH/$CHCl_3$ mixture, filtered through 0.45 μm PTFE filters. The volume injected was 10 μL. Standard solutions for the quantification of all sapogenins were prepared at a concentration of 0.35 mg/mL in methanol. Identification was carried out with regards to retention time and spectra matching. Once spectra matching succeeded, results were confirmed by spiking with the respective standard to achieve a complete identification utilizing the so-called peak purity test. The peak which was not fulfilling these requirements was not quantified. Quantification was performed by external calibration with the standard. Sapogenins yield in analyzed samples was calculated and expressed as mg component/$g_{DM}$ (DM, dry mass of fenugreek seeds).

### Preliminary consideration of SC $CO_2$ density of SFE

SC $CO_2$ density influence, determined by applied pressure and temperature process parameters on obtained extract was considered in preliminary tests. The content of diosgenin, sarsapogenin, protodioscin, oleanolic and ursolic acid under applied conditions was investigated. The yield of target compounds in obtained extracts was considered through the density of $CO_2$. The yield of steroidal sapogenins in obtained extracts was compared and used for further analysis in the influence of different working conditions.

### Response surface methodology and CCRD

RSM and CCRD were used to study the effects of process conditions of SC-$CO_2$ through pressure, temperature and duration of extraction (consumption of SC-$CO_2$) as well as to define interactions among these working conditions on steroidal sapogenins yield in the extract. A similar procedure was applied in previous researches [28,32]. The experimental design for this study was a three-factor central composite rotatable design with 20 individual experimental points. Three independent factors that influenced the extraction yield and content of diosgenin, sarsapogenin, protodioscin, ursolic and oleanolic acid, as steroidal sapogenins, were pressure, temperature, and duration of extraction.

Second-order polynomial equations were used to present effects of independents coded variables ($k$ = 3) as pressure ($X_1$), temperature ($X_2$) and consumption of $CO_2$ ($X_3$) and to represent the extraction yield ($Y_1$) and content of diosgenin ($Y_2$), sarsapogenin ($Y_3$), protodioscin ($Y_4$), ursolic acid ($Y_5$), oleanolic acid ($Y_6$) as affected variables through the response, as presented by the general equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \qquad (1)$$

Matlab 2014 version software was used for analysis and application of RSM model through CCRD composite design to present the influence of independent variables as pressure, temperature and duration of the process on extract yield and content of steroidal sapogenins. The influence of process parameters was presented through regression coefficients fitting the second-order polynomial equations, either by plotted response 3D surfaces. According to model parameters presenting the influence of process conditions on target compounds in obtained extracts by supercritical extraction, optimization of the process could be performed.

Experimental design for the applied model was performed following coded parameters of pressure (MPa), temperature (°C), and consumption of SC-$CO_2$ ($g_{CO2}/g_{DM}$). Central composite design (CCRD) was created to fit a wide range of process conditions, resulting in 20 experiment runs. Uncoded and coded variables for the CCRD model in the range and limits

are presented in Table 1. A combination of process conditions of pressure and temperature by determined and applied experimental design resulted in applied conditions of experiments in the density of SC $CO_2$ from 791 to 957 kg/m$^3$.

*Table 1. Uncoded and coded levels of independent variables used in the RSM design*

| Coded level | Pressure (MPa, $X_1$) | Temperature (°C, $X_2$) | Amount of $CO_2$ (g $CO_2$/g, $X_3$) |
|---|---|---|---|
| -1.682 | 16.6 | 30 | 11.6 |
| -1 | 20 | 34 | 15 |
| 0 | 25 | 40 | 20 |
| +1 | 30 | 46 | 25 |
| +1.682 | 33.4 | 50 | 28.4 |

### Chemicals and reagents

Diosgenin (>99.0%), sarsapogenin (>99.0%), protodioscin (>99.0%), oleanolic acid (>99.0%), and ursolic acid (>99.0%) as standard in HPLC analysis, were purchased from Sigma-Aldrich. Hexane (>95.0%) was purchased from Sigma-Aldrich. Hydrochloric acid (37%) was purchased from Merck (Darmstadt, Germany).

## RESULTS AND DISCUSSION

### The yield of extract as a function of SC $CO_2$ density

Applied range of process parameters as pressure and temperature performed a range of SC-$CO_2$ density working conditions of experiments. SC $CO_2$ density determined by applied pressure and temperature at every single experimental run was 790-930 kg/m$^3$. A similar procedure was applied in preliminary analysis of the influence of SC $CO_2$ density on the yield of the total extract, obtained from fenugreek seeds, as well as on the determined content of sterols, vitamins E and D in the extract [32], and in diosgenin extraction from fenugreek [28]. The yield of total extract, as well as the content of the target steroidal sapogenins diosgenin, sarsapogenin, protodioscin, ursolic, and oleanolic acid, were determined after the same duration of the extraction process (observed through consumption of the same amount of SC $CO_2$, 20 g $CO_2$/$g_{DM}$). Th determined amounts of diosgenin, sarsapogenin, protodioscin, as well as amounts of ursolic and oleanolic acid in obtained extracts, by applied conditions of a wide range of SC-$CO_2$ density, are shown in Figure 1. As the determined content of ursolic acid in obtained extracts was remarkably higher in comparison to the content of other sapogenins, the results for ursolic acid were presented in a different *y*-scale in Figure 1. Earlier performed research revealed the presence of ursolic acid found in hydroalcoholic extracts of fenugreek [16]. To the best of our knowledge, presented for the first time in scientific literature, our results indicated that the SC extraction conducted under the applied conditions enabled the extract rich in ursolic acid.

From data shown in Figure 1, the same tendency observed of an increment of steroidal sapogenins contents was following the rising SC $CO_2$ density. The highest content of steroidal sapogenins was detected when SC $CO_2$ density ranged from 840 to 900 kg/m$^3$. The maximum of steroidal content was found to be when 880 kg/m$^3$ SC $CO_2$ density was applied. The amount of diosgenin, sarsapogenin and protodioscin at density of 880 kg/m$^3$ was 0.61, 0.22 and 0.49 mg/$g_{DM}$, respectively.

The content of ursolic and oleanolic acid, following rising values of SC $CO_2$ density, was not as sig-

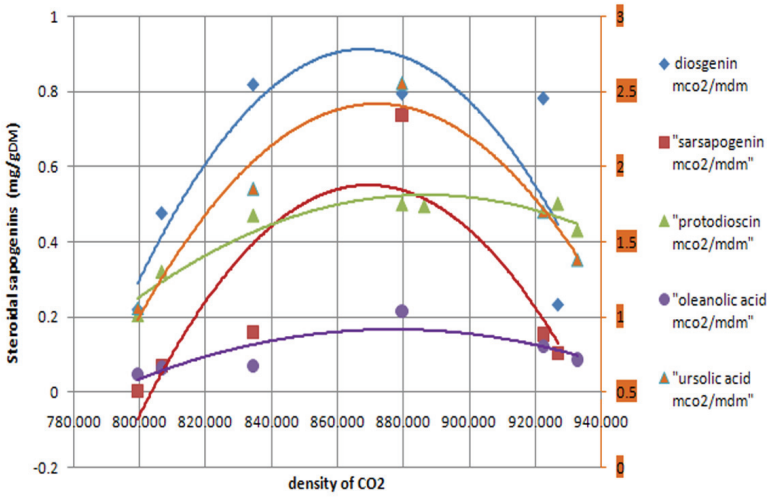


*Figure 1. Steroidal sapogenins content in obtained extracts of fenugreek determined on a wide range of SC $CO_2$ density.*

nificant, as in the case of other steroidal sapogenins. The rising of SC $CO_2$ density was followed by a slight growth of ursolic and oleanolic acid content. The highest yield of oleanolic acid was from 820-920 kg/m$^3$, with maximum content achieved when the SC $CO_2$ density was around 880-900 kg/m$^3$, while the highest content of ursolic acid was from 850-930 kg/m$^3$ with a maximum at 900 kg/m$^3$.

On the performed preliminary test studies, using the different density of SC $CO_2$, working conditions of SC extraction were chosen, as a wide range of pressure, temperature, and consumption of SC $CO_2$. Chosen parameters of SC extraction were coded for application of CCRD model in process optimization.

The experimental set was created to run and investigate conditions from the central point of preliminary studies, enabling it to achieve the highest content of steroidal sapogenins. The obtained amount of extract isolated from fenugreek seeds and the content of steroidal sapogenins (mg/$g_{DM}$), obtained by 20 experimental runs in the range of investigated values of SCE working conditions are presented in Table 2.

## RSM-CCRD analysis

The central composite design was applied to investigate the influence of process parameters through three independent values: pressure, temperature, and consumption of SC $CO_2$. The study was applied by 20 experiment runs with a wide variety of process parameters. Variation of investigated parameters resulted in a different yield of obtained extracts and contents of specific compounds in them. The yield of extract and content of steroidal sapogenins, in correlation to applied condition by the RSM model, have presented influence, interaction, and significance of process conditions. The results of applied process conditions on the achieved yield and content of steroidal sapogenins were investigated by the RSM model. RSM model examined the correlation to fit the quadratic equation. Analysis of variance (ANOVA) test was performed defining the fitting of the obtained correlation to an applied quadratic function. Significance and interaction of process parameters on the yield were investigated and presented by the regression coefficient obtained in the applied model function. Linear and quadratic effects of process parameters, as independent coded variables, were presented through equations which can be formed from the regression coefficient presented in Table 3, in the form of an equation:

*Table 2. Experimental design for optimization of sapogenins content*

| | Independent variables | | | | | | Dependent variables | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Coded variables | | | Uncoded variables | | | Extract yield ($g_{EX}/g_{dm}$) | Diosgenin (mg/g dry material) | Protodioscin (mg/g dry material) | Sarsapogenin (mg/g dry material) | Oleanolic acid (mg/g dry material) | Ursolic Acid (mg/g dry material) |
| | $X_1$ | $X_2$ | $X_3$ | Pressure (MPa) | Temperature (°C) | Amount of $CO_2$ (g $CO_2$/g) | | | | | | |
| 1 | 1 | 1 | 1 | 30 | 46 | 25 | 0.0848 | 0.6995 | 0.2943 | 0.2314 | 0.1213 | 1.8657 |
| 2 | 1 | 1 | -1 | 30 | 46 | 15 | 0.0724 | 0.5295 | 0.6991 | 0.2145 | 0.1121 | 1.5125 |
| 3 | 1 | -1 | 1 | 30 | 34 | 25 | 0.0772 | 0.5532 | 0.4197 | 0.1633 | 0.1102 | 1.5637 |
| 4 | 1 | -1 | -1 | 30 | 34 | 15 | 0.0564 | 0.5114 | 0.4439 | 0.1752 | 0.0632 | 1.1983 |
| 5 | -1 | 1 | 1 | 20 | 46 | 25 | 0.0578 | 0.4958 | 0.3293 | 0.1301 | 0.0721 | 1.2198 |
| 6 | -1 | 1 | -1 | 20 | 46 | 15 | 0.0428 | 0.4588 | 0.3126 | 0.0135 | 0.0558 | 0.7031 |
| 7 | -1 | -1 | 1 | 20 | 34 | 25 | 0.0577 | 0.5186 | 0.4128 | 0.0998 | 0.0741 | 1.2659 |
| 8 | -1 | -1 | -1 | 20 | 34 | 15 | 0.0344 | 0.3624 | 0.0473 | 0.1435 | 0.0453 | 0.9894 |
| 9 | 1.682 | 0 | 0 | 33.4 | 40 | 20 | 0.0677 | 0.2345 | 0.5036 | 0.1052 | 0.0788 | 1.2445 |
| 10 | -1.682 | 0 | 0 | 16.6 | 40 | 20 | 0.0262 | 0.2215 | 0.2045 | 0.0058 | 0.0489 | 1.0591 |
| 11 | 0 | 1.682 | 0 | 25 | 50 | 20 | 0.0746 | 0.8183 | 0.4721 | 0.1615 | 0.0702 | 1.8531 |
| 12 | 0 | -1.682 | 0 | 25 | 30 | 20 | 0.0662 | 0.7821 | 0.3514 | 0.1562 | 0.1209 | 1.6995 |
| 13 | 0 | 0 | 1.682 | 25 | 40 | 28.41 | 0.0765 | 0.7678 | 0.3321 | 0.4148 | 0.1431 | 1.6285 |
| 14 | 0 | 0 | -1.682 | 25 | 40 | 11.59 | 0.0525 | 0.4421 | 0.3759 | 0.4032 | 0.0713 | 1.1951 |
| 15 | 0 | 0 | 0 | 25 | 40 | 20 | 0.0785 | 0.7895 | 0.4948 | 0.7385 | 0.2159 | 2.5598 |
| 16 | 0 | 0 | 0 | 25 | 40 | 20 | 0.0758 | 0.7859 | 0.5046 | 0.7355 | 0.2192 | 2.5564 |
| 17 | 0 | 0 | 0 | 25 | 40 | 20 | 0.0752 | 0.7971 | 0.5071 | 0.739 | 0.2104 | 2.5414 |
| 18 | 0 | 0 | 0 | 25 | 40 | 20 | 0.0761 | 0.8056 | 0.5072 | 0.7386 | 0.2153 | 2.5516 |
| 19 | 0 | 0 | 0 | 25 | 40 | 20 | 0.0754 | 0.7912 | 0.5019 | 0.7391 | 0.2083 | 2.5544 |
| 20 | 0 | 0 | 0 | 25 | 40 | 20 | 0.0759 | 0.7996 | 0.4956 | 0.7351 | 0.2116 | 2.5579 |

*Table 3. Significance of process parameters in steroidal sapogenins content in extracts from fenugreek, presenting correlation of fitted model for investigation*

| Variable | Diosgenin yield (mg/g dry material) | | | Protodioscin yield (mg/g dry material) | | | Sarsapogenin yield (mg/g dry material) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Regresssion coefficients | Standard error | *p*-Value | Regresssion coefficients | Standard error | *p*-Value | Regresssion coefficients | Standard error | *p*-Value |
| Constant | +0.80 | 0.21 | < 0.0001 | +0.50 | 3.263E-003 | < 0.0001 | +0.74 | 0.010 | < 0.0001 |
| $X_1$ | +0.035 | 0.014 | 0.0295 | +0.092 | 2.165E-003 | < 0.0001 | +0.041 | 6.836E-003 | 0.0001 |
| $X_2$ | +0.022 | 0.014 | 0.1450 | +0.038 | 2.165E-003 | < 0.0001 | +1.216E-003 | 6.836E-003 | 0.8623 |
| $X_3$ | +0.070 | 0.014 | 0.0005 | -8.821E-003 | 2.165E-003 | 0.0022 | +7.133E-003 | 6.836E-003 | 0.3214 |
| $X_1^2$ | -0.20 | 0.013 | < 0.0001 | -0.051 | 2.107E-003 | < 0.0001 | -0.25 | 6.655E-003 | < 0.0001 |
| $X_2^2$ | -8.489E-004 | 0.013 | 0.9510 | -0.031 | 2.107E-003 | < 0.0001 | -0.21 | 6.655E-003 | < 0.0001 |
| $X_3^2$ | -0.070 | 0.013 | 0.0004 | -0.051 | 2.107E-003 | < 0.0001 | -0.12 | 6.655E-003 | < 0.0001 |
| $X_1X_2$ | +0.011 | 0.018 | 0.5444 | -6.500E-003 | 2.828E-003 | 0.0444 | +0.026 | 8.932E-003 | 0.0159 |
| $X_1X_3$ | +2.325E-003 | 0.018 | 0.9003 | -0.10 | 2.828E-003 | < 0.0001 | -8.488E-003 | 8.932E-003 | 0.3644 |
| $X_2X_3$ | +1.125E-003 | 0.018 | 0.9516 | -0.091 | 2.828E-003 | < 0.0001 | -0.061 | 8.932E-003 | 0.0245 |
| Lack of fit | 0.026 | | | 0.37 | | | 0.16 | | |
| $R^2$ | 0.9654 | | | 0.9982 | | | 0.9958 | | |
| Adj $R^2$ | 0.9342 | | | 0.9966 | | | 0.9920 | | |

$$Y_i = \beta_0 + \beta_{1,i}X_1 + \beta_{2,i}X_2 + \beta_{3,i}X_3 + \beta_{11,i}X_1^2 + \beta_{22,i}X_2^2 + \\ + \beta_{33,i}X_3^2 + \beta_{12,i}X_1X_2 + \beta_{13,i}X_1X_3 + \beta_{23,i}X_2X_3 \quad (2)$$

where $Y$ presents the yield of extract, $i$ content of diosgenin, sarsapogenin, protodioscin, ursolic and oleanolic acid, as steroidal sapogenins (mg/g dry basis fenugreek seed), $X_1$, coded value of pressure, $X_2$ coded value of temperature, and $X_3$ coded value of amount of used SC $CO_2$, as the duration of the extraction process. The results of applied ANOVA tests examined the fitting model to the relation of the obtained process on the achieved yield, investigating the significance of positive and negative effects of the interaction of different parameters as well. The applied model enabled the examination of the results of relations for obtained steroidal sapogenins in extracts (Table 3).

Based on the determined coefficient $R^2$, and the determined adjusted coefficient $R^2_{adj}$, the applied model was considered suitable to fit predicted and experimental data. On the other side, lack of fit, as another coefficient of statistical analysis revealing the fit of the model, significant with *p*-values less than 0.05, indicated that the derived model equation might not be adequate to represent the observed response [33]. By indications of the non-adequate fitting of the model to correlated data, an explanation would be in calculating the equation of the lack of fit, which was calculated by dividing the lack of fit mean square by the pure error mean square. As the pure error mean square was small, it resulted in a greater lack of fit value and smaller *p*-value. Analyzing the residual coefficients, determining the correlation within predicted and obtained data, the model was shown to enable the adequate fitting without showing any deviations. Therefore, based on observation of all presented statistical regression coefficients, the applied model was relevant in description and prediction of steroidal sapogenins content in fenugreek extracts, pointing out the valuable correlation between applied process parameters as pressure, temperature, and consumption of $CO_2$ (independent variables) and dependent variables as the yield of target compounds.

$R^2$ values of the fitting model regarding the applied correlation of process parameters for diosgenin, sarsapogenin and protodioscin (0.98, 0.99 and 0.99, respectively) were greater than 0.8, indicating that the model fitted the data.

In the case of diosgenin, both linear and quadratic terms of pressure and SC $CO_2$ revealed a significant influence on the content, while the interactions between pressure, temperature, and the amount of $CO_2$ were not significant. On the other hand, in the case of protodioscin, pressure, temperature and amount of $CO_2$ appeared to be significant in linear and quadratic form, and the interactions between all parameters were shown to be significant. Linear and quadratic terms of pressure were significant, while the quadratic term of temperature and amount of $CO_2$ significantly influenced the content of sarsapogenin in obtained extracts, and the significant interaction of pressure, temperature and amount of $CO_2$ were revealed.

Values of $R^2$, regarding the fitting model in case of the yield of obtained extracts, the content of ursolic

and oleanolic acid, indicated a good model correlation to obtained data, presented in Table 4.

Linear and quadratic terms of pressure, temperature and amount of $CO_2$ appeared to influence significantly the extract yield, while interactions between pressure and temperature, and temperature and amount of $CO_2$ were significant as well.

The ursolic and oleanolic acid content in obtained extract was influenced significantly by pressure and amount of $CO_2$ in linear and quadratic terms, while temperature exhibited the significant influence in the quadratic term. A significant interaction was observed in pressure-temperature terms regarding the ursolic content, while in the case of oleanolic determination interactions were not observed.

## Analysis of response surface

RSM analysis of process parameters on the content of steroidal sapogenins was presented through 3D plotted surfaces in Figures 2-6.

The 3-D response of the applied model visualized the influence of process parameters on the yield of steroidal sapogenins, as well as the interaction of process conditions and summarized their effect on obtaining the maximum yield of target compounds.

The influence of pressure and consumption of $CO_2$ were significant in linear and quadratic terms on diosgenin yield, as observed in preliminary investigation [28], while any interaction between process conditions was not observed in diosgenin content. Increasing pressure during the process significantly increased diosgenin yield in obtained extracts until

*Table 4. Significance of process parameters in extract yield from fenugreek and sapogenins content in extracts from fenugreek, presenting correlation of fitted model for investigation*

| Variable | Oleanolic acid yield (mg/g dry material) | | | Ursolic acid yield (mg/g dry material) | | | Extract yield | | |
|---|---|---|---|---|---|---|---|---|---|
| | Regresssion coefficients | Standard error | *p*-Value | Regresssion coefficients | Standard error | *p*-Value | Regresssion coefficients | Standard error | *p*-Value |
| Constant | +0.21 | 6.6E-003 | < 0.0001 | +2.56 | 0.053 | < 0.0001 | +0.076 | 6.7E-004 | < 0.0001 |
| $X_1$ | +0.015 | 4.4E-003 | 0.0060 | +0.17 | 0.035 | 0.0008 | +0.012 | 4.4E-004 | < 0.0001 |
| $X_2$ | -1.2E-003 | 4.4E-003 | 0.7868 | +0.040 | 0.035 | 0.2814 | +3.3E-003 | 4.4E-004 | < 0.0001 |
| $X_3$ | +0.016 | 4.4E-003 | 0.0043 | +0.16 | 0.035 | 0.0008 | +8.1E-003 | 4.4E-004 | < 0.0001 |
| $X_1^2$ | -0.053 | 4.3E-003 | < 0.0001 | -0.51 | 0.034 | < 0.0001 | -0.010 | 4.3E-004 | < 0.0001 |
| $X_2^2$ | -0.042 | 4.3E-003 | < 0.0001 | -0.29 | 0.034 | < 0.0001 | -1.8E-003 | 4.3E-004 | 0.0016 |
| $X_3^2$ | -0.037 | 4.3E-003 | < 0.0001 | -0.42 | 0.034 | < 0.0001 | -3.9E-003 | 4.3E-004 | < 0.0001 |
| $X_1X_2$ | +6.4E-003 | 5.7E-003 | 0.2910 | +0.12 | 0.046 | 0.0263 | +1.8E-003 | 5.8E-004 | 0.0091 |
| $X_1X_3$ | +1.3E-003 | 5.7E-003 | 0.8150 | -9.3E-003 | 0.046 | 0.8419 | -6.3E-004 | 5.8E-004 | 0.3018 |
| $X_2X_3$ | -6.2E-003 | 5.7E-003 | 0.3018 | +0.028 | 0.046 | 0.5455 | -2.0E-003 | 5.8E-004 | 0.0051 |
| Lack of fit | 2.5E-003 | | | 0.17 | | | 2.0E-005 | | |
| $R^2$ | 0.9674 | | | 0.9779 | | | 0.9943 | | |
| Adj $R^2$ | 0.9381 | | | 0.9581 | | | 0.9893 | | |

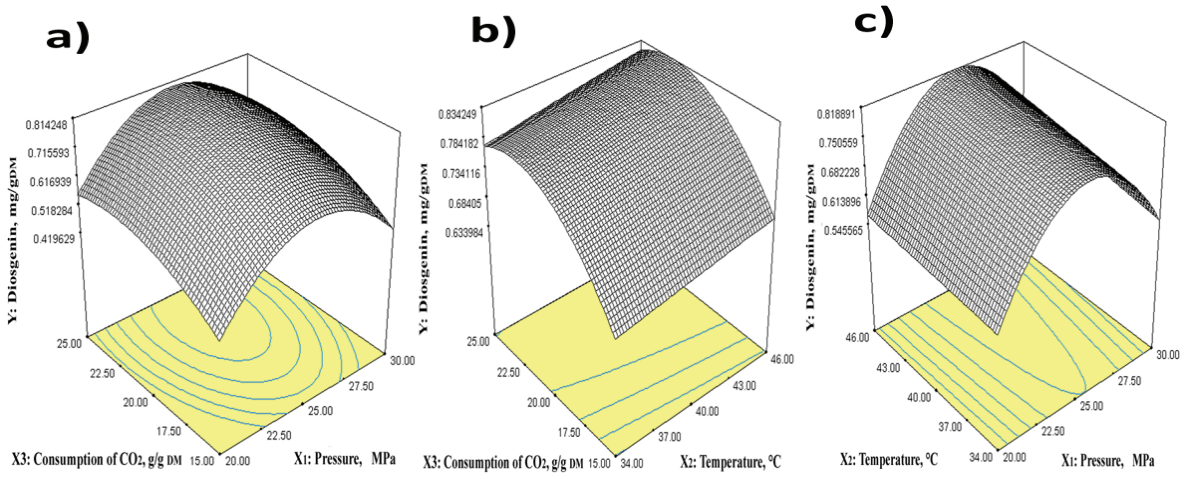


*Figure 2. 3D surface plot of process parameters influence on the extraction yield of diosgenin.*

the value of 25 MPa, when the further increase of pressure resulted in decreased yield, presented in 3D surface plots in Figure 2a. The increase of consumption of $CO_2$ as the duration of the process resulted in a significant increase in the yield until certain value, wherefrom further increase led to a longer time of the extraction process than to increasing the yield over the already constant value, presented in Figure 2b. The temperature had no significant influence, although from a certain value with rising pressure above the value of 25 MPa, rising temperature resulted in a higher decrease of the yield of diosgenin as a result of reducing solvent power, presented in Figure 2c. In this study, diosgenin content, as shown in a previous study [28], confirmed that the increase of temperature did not result in higher vapor pressure of diosgenin, according to its molar weight, and that by further increase above the value of 40 °C, the increase of temperature led to a decrease of $CO_2$ density and reducing solvent power. On the other hand, the effect of pressure on diosgenin content was significant, proving that increasing solvent density by an applied increase of pressure resulted in higher extraction potential.

Besides, the temperature had a significant effect on the estimation of protodioscin content in obtained extracts, positive in linear term and negative in the quadratic term, presented in Figure 3b. In this case, temperature influenced higher vapor pressure more than reducing solvent power, resulting in a higher yield of protodioscin, presented in Figure 3c. Beyond the significant influence of pressure, positive in linear and negative in the quadratic term, through increasing extraction potential by larger solvent density, consumption of $CO_2$ as the duration of process extraction, was significant both as a negative effect in the linear and quadratic term, presented in Figure 3a. Following protodioscin content, significant interactions between all process parameters were observed. Interactions of process parameters were all negative, not contributing to the higher content of protodioscin in obtained extracts (estimated through negative regression coefficients). Interactions between all processes parameters were significant in estimating protodioscin content presented in Figure 3. Interactions can be observed on 3D plots, presenting obvious sharp increase in protodioscin content by increasing process duration and pressure until reaching the value of 23 MPa, while by further increase of pressure further increase of consumption yield was observed only for lower values of $CO_2$ consumption, presenting the effect of gaining higher content by higher pressure and shorter process duration. A similar effect was observed in the case of interaction between consumption of $CO_2$ and temperature, resulting in a higher yield by increasing the temperature and consumption of $CO_2$ until the value of 37 °C, when the yield increased with further increasing of temperature, with lower consumption of $CO_2$ (shorter process extraction). With higher temperature or pressure values, higher yield could be achieved if the duration of the process was shorter, as in the case of increased process duration; yield of protodioscin would decrease significantly either on higher values of pressure or temperature. Increasing pressure and temperature resulted in increasing the content of protodioscin for any values, which was not observed in the case of diosgenin and sarsapogenin.

Sarsapogenin yield was significantly influenced by positive linear pressure and negative quadratic terms of pressure and negative quadratic term of $CO_2$ consumption, while the positive linear term of $CO_2$

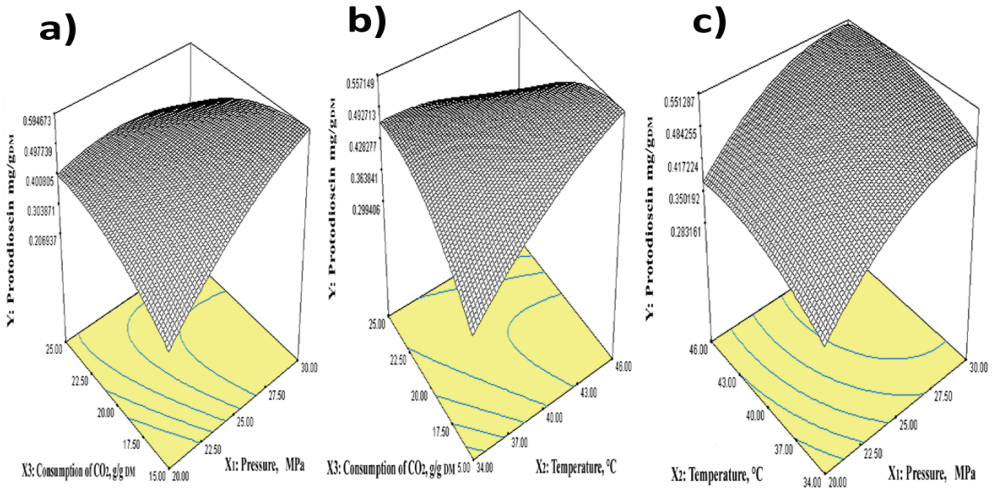


*Figure 3. 3D surface plots of process parameters influence on protodioscin extraction yield.*

consumption was not significant, presented in Figure 4a. Quadratic term of temperature was significant as well as in negative effect, while the linear term of temperature was not significant for sarsapogenin content, presented in Figure 4b. Increasing the pressure was significant on achieved sarsapogenin yield leading to higher solvent power by larger density, while the quadratic significance of temperature showed rising vapor pressure of sarsapogenin until some value, wherefrom solvent reducing power overwhelmed the influence on sarsapogenin content, decreasing the yield in obtained extracts, presented in Figure 4c. Increasing in the yield by increasing consumption of $CO_2$ until the value of 20 $g/g_{DM}$ and pressure until the value of 25 MPa was significant, but further increasing of pressure and temperature above determined values resulted in a decrease in yield. A similar tendency was noticed in case of the influence of $CO_2$ consumption and temperature, where the yield of sarsapogenin was increased by increasing both parameters until values of 40 °C and 20$g/g_{DM}$, wherefrom by a further increase of process parameters a significant decrease in the yield was examined. Pressure and temperature exhibited an increase of yield until some optimal value of 25 MPa and 40 °C, and a further increase of both parameters would result in yield decrease. Interactions between pressure and temperature, and temperature and the amount of $CO_2$ were examined and turn out to have a positive effect to the yield.

According to the different influence of process parameters, their interactions and tendency relation on diosgenin, protodioscin and sarsapogenin content, all model observation was estimated to understand and explain the influence of process parameters and to find optimal conditions with the aim to achieve maximum yield for all of them.

3D plots present the extract yield influenced by process parameters: pressure, temperature and consumption of $CO_2$. The highest extract yield was achieved by the highest pressure and temperature, although growth was slightly low to constant from 25 MPa and 40 °C. The increasing temperature had a positive effect for all the duration of the process, explaining the positive effect on the extraction process by increase through vapor pressure of compounds, and a slightly low effect in reduction of solvent power by a decrease in density. Extract yield was increased by increasing pressure and temperature, although from the pressure of 25 MPa, the increase was not significant, becoming constant. For higher temperatures, increasing pressure led to a higher yield of extract, which was not the case for lower temperatures.

In the case of ursolic and oleanolic acid yield, the influence of process parameters was common, as yield was increased by increasing the values of parameters, until values of 20 MPa, 40 °C and 20 $g/g_{DM}$ of $CO_2$ consumption, wherefrom further rising of any value led to a decrease in yield, presented in Figures 5 and 6, respectively. In the examination of ursolic acid content in obtained extracts, pressure exhibited a significant positive effect in the linear term in favor of extraction potential by increasing solvent density power, while the significant but negative effect of pressure was observed in the quadratic term, presented in Figure 5a and b. The temperature had no significant effect in the linear term although the positive effect was observed, while in the quadratic term the significant negative effect of temperature was estimated showing that the increase of temperature resulted in reducing solvent power with increasing overhead vapor pressure of ursolic acid, presented in Figure 5c. Positive interaction between process parameters of pressure and temperature was examined,

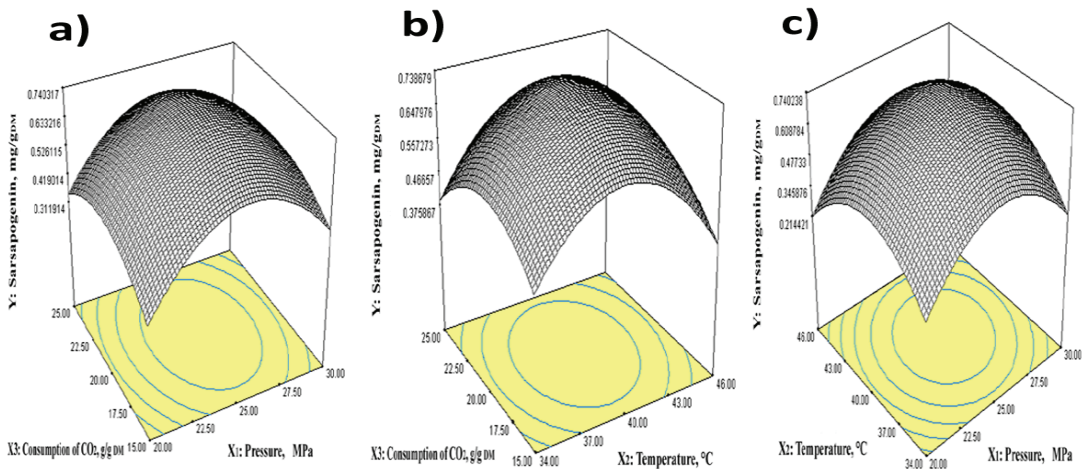


*Figure 4. 3D surface plots of process parameters influence on sarsapogenin extraction yield.*

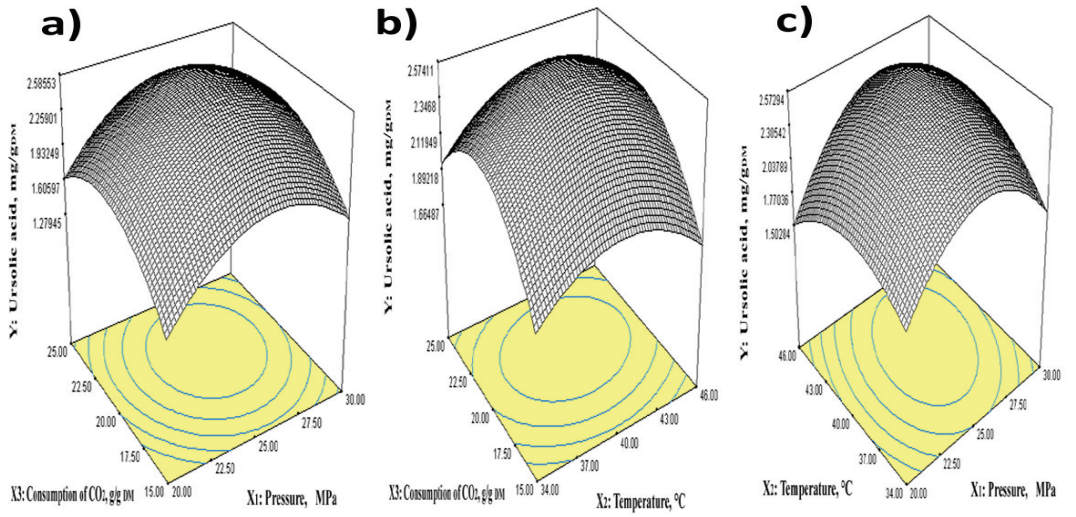


*Figure 5. 3D surface plots of process parameters influence on ursolic acid extraction yield.*

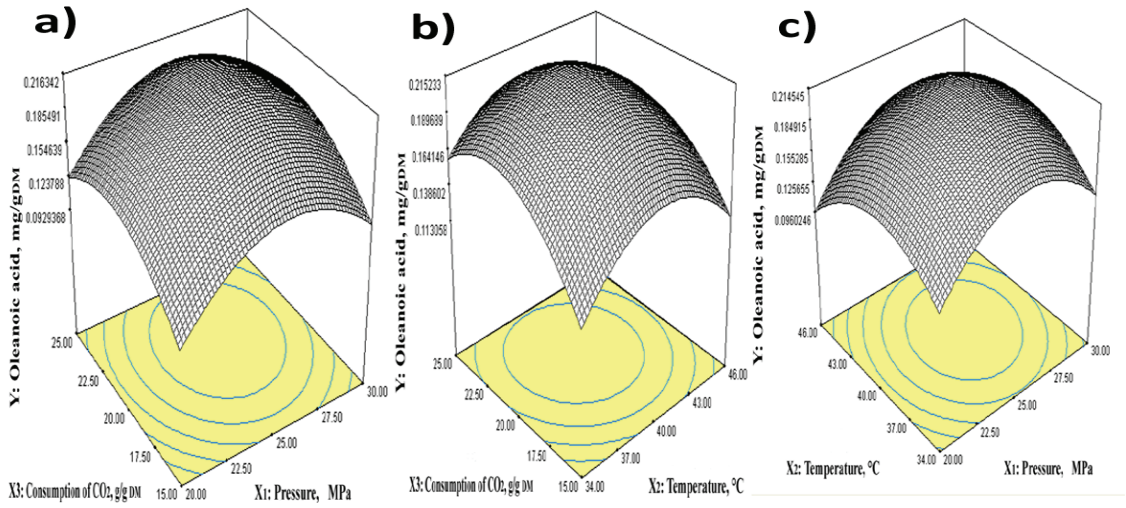


*Figure 6. 3D surface plots of process parameters influence on oleanolic acid extraction yield.*

significantly and positively influencing ursolic content in obtained extracts.

Oleanolic content in obtained extracts was not significantly influenced by interactions among process parameters following the example of ursolic acid. The effect of pressure was estimated to be positive in the linear term, presenting increase of solvent density and greater extraction potential, while the quadratic term of pressure appeared to be negative on oleanolic content, presented in Figure 6c. Temperature influenced oleanolic acid content significantly in a quadratic term as a negative effect, showing that reducing solvent power decreased oleanolic acid extraction, presented in Figure 6b. Process duration, observed through consumption of $CO_2$, significantly influenced oleanolic acid content in a positive linear term and a negative quadratic term, presented in Figure 6a.

## Optimization

In addition to applied RSM analysis of supercritical extraction process parameters affecting the yield of steroidal sapogenins, applied CCRD design and fitting model to obtained results, optimization was estimated. According to the obtained analysis of SC process influence on the yield of target compounds, optimal process parameters for maximal yield were determined. Optimal process conditions were determined and presented, examining achieved values of extract and steroidal sapogenins content obtained by set conditions. By applied investigation of process parameters on the extraction of the sapogenin group of compounds from fenugreek, it was determined that 25.87 MPa, 40.51 °C and 20.62 $g/g_{DM}$ were optimal conditions for obtaining the extract with the highest yield of diosgenin, sarsapogenin, protodioscin, olea-

nolic and ursolic acid. The achieved content of steroidal sapogenins was 0.805 mg/$g_{DM}$ diosgenin, 0.514 mg/$g_{DM}$ protodioscin and 0.736 mg/$g_{DM}$ sarsapogenin, while the maximal yield of oleanolic and ursolic acid was 0.216 and 2.587 mg/$g_{DM}$, respectively. The obtained highest yield of an extract rich in sapogenins from fenugreek was estimated to be 0.079 g/$g_{DM}$.

## CONCLUSION

In this research, SC extraction was applied to obtain a maximal yield of extracts with the highest content of steroidal sapogenins, as a general group of sapogenin compounds in fenugreek seed. RSM and CCRD analysis model were applied to optimize and study the influence of process parameters on the yield of obtained extracts and content of the target steroidal sapogenins: diosgenin, sarsapogenin, and protodioscin, as well as oleanolic and ursolic acid. The aim was to study those relations and to find optimal conditions to achieve maximal contents for all sapogenins in obtained extracts. The optimal conditions for obtaining maximal extract yield, as well as the content of steroidal sapogenins were defined by temperature and pressure, which defined wide range values of SC $CO_2$ density and consumption of SC $CO_2$, presenting the actual extraction process time. Optimal process conditions determined for achieving maximal extract yield and the content of steroidal sapogenins are: pressure of 24.73 MPa, temperature of 38.15 °C and consumption of SC $CO_2$ of 19.24 g/$g_{DM}$. The best conditions to achieve a maximal yield of an extract with the highest content of steroidal sapogenins appeared to be SC $CO_2$ density of 885.47 kg/$m^3$.

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ALEKSANDRA BOGDANOVIC[1]
VANJA TADIC[2]
SLOBODAN PETROVIC[1]
DEJAN SKALA[1]

[1]Univerzitet u Beogradu, Tehnološko-metalurški fakultet, Karnegijeva 4, Karnegijeva 4, 11120 Beograd, Srbija
[2]Institut za medicinska istraživanja "Dr Josif Pančić", Tadeuša Košćuška 1, 11000 Beograd, Srbija

NAUČNI RAD

# NATKRITIČNA EKSTRAKCIJA STEROIDNIH SAPOGENINA UGLJENIK(IV)-OKSIDOM IZ GRČKOG SEMENA (*Trigonella foenum-graecum* L.)

*Natkritična ekstrakcija ugljenik (IV)-oksidom je primenjena za ekstrakciju grčkog semena (Trigonella foenum-graecum L., Fabaceae) u cilju definisanja optimalnih procesnih uslova kako bi se ostvario maksimalan prinos steroidnih sapogenina. Centralno kompozitno rotirajući dizajn (CCRD) u kombinaciji sa metodologijom odzivnih površina (RSM) je primenjen kako bi se definisali optimalni uslovi shodno primenjenim procesnim uslovima, kao što su, uticaj i interakcija pritiska, temperature i vremena ekstrakcije izražene preko količine utrošenog ugljenik(IV)-oksida. Optimizacijom procesa natkritične ekstrakcije steroidnih sapogenina iz grčkog semena, determinisano je da procesni uslovi pritiska od 24,73 MPa, temperature od 38,2 °C i količina utrošenog NK $CO_2$ od 19,24 g/$g_{BM}$ predstavljaju najbolje uslove sa ostvarivanje maksimalnog prinosa ekstrakta sa najvećim sadržajem sapogenina. Primenjeni uslovi optimalnih vrednosti pritiska i temperature, definišu gustinu NK $CO_2$ od 885,47 kg/$m^3$, koja omogućava ekstrakciju u najvećem prinosu i najvećem sadržaju sapogenina u dobijenom ekstraktu iz grčkog semena. Ostvarena vrednost maksimalnog prinosa ekstrakta iz grčkog semena pri primenjenim optimalnim vrednostima procesa je bila 0,073g/$g_{BM}$ sa ostvarenim maksimalnim sadržajem sapogenina od 0,774 mg/$g_{BM}$ diosgenina, 0,477 mg/$g_{BM}$ protodioscina, 0,713 mg/$g_{БМ}$ sarsapogenina i 0,205 mg/$g_{BM}$ oleanolne i ursolne kiseline sa značajnim sadržajem od 2,475 mg/$g_{BM}$.*

*Ključne reči: sapogenini, grčko seme, optimizacija.*