Hrana u zdravlju i bolesti, znanstveno-stručni časopis za nutricionizam i dijetetiku (2020) 9 (1) 30-39
Food in Health and Disease, scientific-professional journal of nutrition and dietetics (2020) 9 (1) 30-39

OPTIMIZATION OF ETHANOL/WATER SOLVENT EXTRACTION OF BIOACTIVE COMPONENTS ORIGINATING FROM INDUSTRIAL HEMP (*Cannabis sativa* L.)

Adela Šain, Nikolina Matešić, Tamara Jurina*, Ana Jurinjak Tušek, Maja Benković, Davor Valinger, Jasenka Gajdoš Kljusurić

University of Zagreb, Faculty of Food Technology and Biotechnology, Pierottijeva 6, 10000 Zagreb, Croatia

original scientific paper

Summary

Hemp (*Cannabis sativa* L.) contains a wide range of biocompounds with different beneficial properties such as anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic and antioxidative. Response Surface Methodology (RSM) coupled with Box-Behnken design (BBD) was applied to determine the influence of extraction temperature, liquid to solid ratio, extraction time, rotational speed and ethanol/water solvent ratios at three levels on the solid-liquid extraction of the bioactives from the hemp (flowers, leaves, seeds, stems). Based on the obtained results, liquid to solid ratio, temperature and ethanol/water solvent ratio had statistically significant effects on the total polyphenolic content (TPC), while extraction time and rotational speed had no influence on the TPC extraction. Regarding antioxidant activity (AOX) determined by the DPPH method, only liquid to solid ratio had a statistically significant effect. Liquid to solid ratio, ethanol/water solvent ratio, temperature and rotational speed significantly influenced AOX determined by the FRAP method. According to BBD, the optimum extraction conditions were as follows: extraction temperature 45 °C, liquid to solid ratio 30 mL/g, extraction time 25 min, rotational speed 500 rpm, ethanol/water solvent ratio 25%. RSM coupled with a BBD model was shown to be effective for optimization the solid-liquid extraction of hemp.

Keywords: hemp (*Cannabis sativa* L.), solid-liquid extraction, optimization, bioactives

Introduction

Hemp (*Cannabis sativa* L.) is a herbaceous plant of the *Cannabaceae* family that grows in diverse climates all over the world (Kostić et al., 2013). It has been cultivated for a long time for medicinal and food purposes, together with its use as a source of textile fibre. The genus *Cannabis* has been divided into three main species: (i) *Cannabis sativa* L., a fibre-type one, rich of cannabidiol (CBD), (ii) *Cannabis indica* Lam., a drug-type one, characterised by high levels of the psychoactive compound Δ^9-tetrahydrocannabinol (Δ^9-THC) and (iii) *Cannabis ruderalis* Janisch with intermediate properties (Appendino et al., 2011; Thomas and ElSohly, 2015; Hartsel et al., 2016; Pellati et al., 2018).

Several chemical classes that have been identified in *C. sativa* are: terpenes, carbohydrates, fatty acids and their esters, amides, amines, phytosterols, phenolic compounds and cannabinoids. Among them, cannabidiol represents the most valuable one from the pharmaceutical point of view, since it has been found to possess a high antioxidant and anti-inflammatory activity, together with antibiotic, neuroprotective, anxiolytic, and anticonvulsant properties (Pellati et al., 2018). Regarding phenolic compounds present in *C. sativa*, several flavonoids have been identified, belonging mainly to flavones and flavonols (Pollastro et al., 2018). Most of these compounds have a wide range of different properties such as anti-inflammatory, neuroprotective, anticancer and antioxidative (Andre et al., 2016).

Extraction procedure is a primary step for identification and quantification of chemical compounds from plant materials. Many conventional methods can be used to extract these compounds, such as solid-liquid extraction and heated reflux extraction. In addition, a number of advanced methods, such as ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, accelerated solvent extraction, or high hydrostatic pressure extraction are also applied in the extraction of different compounds (Xu et al., 2017). Among them, solid-liquid extraction represents the most commonly used method for extracting phenolics from various plants. Many parameters, such as solvent concentration, extraction time, temperature, pH, liquid/solid ratio and particle size, may significantly influence the solid-liquid extraction (Radojković et al., 2012; Xu et al., 2017; Tchabo et al., 2018). These parameters should be taken into consideration in order to attain the optimal/highest extraction efficiency (Sifaoui et al., 2016). In order to optimize parameters of extraction process, Response Surface Methodology (RSM) has been widely used. RSM is a statistical method for evaluating multiple

*Corresponding author: tlekic@pbf.hr

process parameters and their interactions using quantitative data, optimizing complex extraction procedures, thus reducing the number of experimental trials required. RSM methodology combines mathematical and statistical techniques for designing experiments, building models, evaluating effects of experimental parameters and searching optimum condition of parameters for desirable responses (Liu et al., 2015). Box-Behnken design (BBD), as a part of RSM, has been generally used in various experiments to investigate the impact of more factors at three levels with the ability of effective evaluation of the coefficients of first- and second-order mathematical models (Bezerra et al., 2008).

Bioactive compounds present in industrial hemp have received more and more attention by the biochemical and nutritional researchers due to their biological activities and health function in health-care food or medicine, especially their antioxidant, anti-ultraviolet radiation, and antibacterial effects (Wang et al., 2014; Cao et al., 2017). These antioxidant activities make this plant important for oil, food, pharmaceutical, cosmetics and fiber industries (Kolodziejczyk et al., 2012; Shahid-ul-Islam et al., 2013; Sarasini and Fiore, 2018). These industries are dependent on various extraction processes. Since many factors can influence the efficiency of antioxidants extractions from the plant matrices, an individual approach for extraction and optimization is required. There is no universal extraction and optimization technique, due to the diversity of bioactive compounds (Silva et al., 2007; Majeed et al., 2016).

Having in mind the above mentioned, the aim of this study was to apply the Response Surface Methodology (RSM) approach, coupled with Box-Behnken design (BBD), to optimize the ethanol/water solvent extraction of bioactive components from industrial hemp considering the following variable extraction parameters: extraction temperature, liquid to solid ratio, extraction time, rotational speed and ethanol/water solvent ratios. Analysis of Variance (ANOVA) was used to evaluate the significance of the extraction parameters on the total polyphenolic content (TPC) and antioxidant activity (AOX) of the obtained hemp extracts.

Materials and methods

Materials

Plant material

Dried industrial hemp (*Cannabis sativa* L.) (flowers, leaves, seeds, stems) was purchased from local producer (OPG Levačić, Prelog, Croatia). The plant material was collected in the north-western part of Croatia (Međimurje), 2018, dried naturally, and the final dry matter content of the lot was 91.03%.

Chemicals and reagents

Folin-Ciocalteu reagent and sodium carbonate were purchased from Kemika (Zagreb, Croatia). Gallic acid (3,4,5-trihydroxybenzoic acid) and iron(II) sulfate heptahydrate were obtained from Aldrich (Sigma–Aldrich, Chemie, Steinheim, Germany). DPPH (2,2-diphenyl-1-picrylhydrazyl) and Trolox (6-hydroxy-2,5,7,8-tetra methylchromane-2-carboxylic acid) was obtained from Fluka (Buchs, Switzerland). Methanol was obtained from J.T. Baker (Deventer, The Netherlands). Hydrochloric acid was obtained from Carlo Erba Reagents (Val de Reuil, France). Acetic acid was purchased from T.T.T. d.o.o. (Novaki, Sv. Nedelja, Croatia). Sodium acetate trihydrate, iron(III) chloride hexahydrate and ethanol (96%) were purchased from Gram-Mol (Zagreb, Croatia). TPTZ (2,4,6-Tris(2-pyridyl)-1,3,5-triazine) was obtained from Sigma (Buchs, Switzerland). Chemicals were of analytical reagent grade.

Methods

Design of experiments

Response Surface Methodology (RSM) was used for investigating the influence of five independent variables (extraction parameters) on the chemical characteristics (total polyphenolic content and antioxidant activity) of the hemp extracts. The experiment was performed using Box-Behnken experimental design. The main parameters affecting solid-liquid extraction including liquid to solid ratio (10, 30, 50 mL/g), ethanol content (0, 25, 50 %), temperature (30, 45, 60 °C), extraction time (5, 25, 45 min) and rotational speed (250, 500, 750 rpm) were selected as independent variables that should be optimized. The resulting experimental design comprised of 46 experiments as shown in Table 1.

Table 1. Response Surface Methodology, coupled with Box-Behnken experimental design

Experiment No.	Liquid to solid ratio (mL/g)	Ethanol content (%)	Temperature (°C)	Time (min)	Rotational speed (rpm)
1	10 (-1)	0 (-1)	45 (0)	25 (0)	500 (0)
2	50 (+1)	0 (-1)	45 (0)	25 (0)	500 (0)
3	10 (-1)	50 (+1)	45 (0)	25 (0)	500 (0)
4	50 (+1)	50 (+1)	45 (0)	25 (0)	500 (0)
5	30 (0)	25 (0)	30 (-1)	5 (-1)	500 (0)
6	30 (0)	25 (0)	60 (+1)	5 (-1)	500 (0)
7	30 (0)	25 (0)	30 (-1)	45 (+1)	500 (0)
8	30 (0)	25 (0)	60 (+1)	45 (+1)	500 (0)
9	30 (0)	0 (-1)	45 (0)	25 (0)	250 (-1)
10	30 (0)	50 (+1)	45 (0)	25 (0)	250 (-1)
11	30 (0)	0 (-1)	45 (0)	25 (0)	750 (+1)
12	30 (0)	50 (+1)	45 (0)	25 (0)	750 (+1)
13	10 (-1)	25 (0)	30 (-1)	25 (0)	500 (0)
14	50 (+1)	25 (0)	30 (-1)	25 (0)	500 (0)
15	10 (-1)	25 (0)	60 (+1)	25 (0)	500 (0)
16	50 (+1)	25 (0)	60 (+1)	25 (0)	500 (0)
17	30 (0)	25 (0)	45 (0)	5 (-1)	250 (-1)
18	30 (0)	25 (0)	45 (0)	45 (+1)	250 (-1)
19	30 (0)	25 (0)	45 (0)	5 (-1)	750 (+1)
20	30 (0)	25 (0)	45 (0)	45 (+1)	750 (+1)
21	30 (0)	25 (0)	45 (0)	25 (0)	500 (0)
22	30 (0)	25 (0)	45 (0)	25 (0)	500 (0)
23	30 (0)	25 (0)	45 (0)	25 (0)	500 (0)
24	30 (0)	0 (-1)	30 (-1)	25 (0)	500 (0)
25	30 (0)	50 (+1)	30 (-1)	25 (0)	500 (0)
26	30 (0)	0 (-1)	60 (+1)	25 (0)	500 (0)
27	30 (0)	50 (+1)	60 (+1)	25 (0)	500 (0)
28	10 (-1)	25 (0)	45 (0)	5 (-1)	500 (0)
29	50 (+1)	25 (0)	45 (0)	5 (-1)	500 (0)
30	10 (-1)	25 (0)	45 (0)	45 (+1)	500 (0)
31	50 (+1)	25 (0)	45 (0)	45 (+1)	500 (0)
32	30 (0)	25 (0)	30 (-1)	25 (0)	250 (-1)
33	30 (0)	25 (0)	60 (+1)	25 (0)	250 (-1)
34	30 (0)	25 (0)	30 (-1)	25 (0)	750 (+1)
35	30 (0)	25 (0)	60 (+1)	25 (0)	750 (+1)
36	10 (-1)	25 (0)	45 (0)	25 (0)	250 (-1)
37	50 (+1)	25 (0)	45 (0)	25 (0)	250 (-1)
38	10 (-1)	25 (0)	45 (0)	25 (0)	750 (+1)
39	50 (+1)	25 (0)	45 (0)	25 (0)	750 (+1)
40	30 (0)	0 (-1)	45 (0)	5 (-1)	500 (0)
41	30 (0)	50 (+1)	45 (0)	5 (-1)	500 (0)
42	30 (0)	0 (-1)	45 (0)	45 (+1)	500 (0)
43	30 (0)	50 (+1)	45 (0)	45 (+1)	500 (0)
44	30 (0)	25 (0)	45 (0)	25 (0)	500 (0)
45	30 (0)	25 (0)	45 (0)	25 (0)	500 (0)
46	30 (0)	25 (0)	45 (0)	25 (0)	500 (0)

Solid-liquid extraction of bioactive components from the hemp

Solid-liquid extraction was performed according to the conditions defined using Box-Behnken experimental design (Table 1). Certain mass of dried hemp material was placed in a 50 mL glass beaker with certain volume of ethanol/water solvent. Extraction experiments were performed using IkaHBR4 digital oil-bath (IKA-WerkGmbH & Co.KG, Staufen, Germany) at defined temperatures, at a specified rotational speed, for a given time. After extraction, samples were filtered through a 100% cellulose filter paper (LLG Labware, Meckenheim, Germany) with 20–25 μm pore size and stored at 4 °C until analyzed (Jurinjak Tušek et al., 2018).

Determination of total polyphenolic content and antioxidant activity

Total polyphenolic content (TPC) of the hemp extracts was determined spectrophotometrically by the Folin-Ciocalteu reagent, according to Singleton and Rossi (1965). All analyses were performed in duplicate and the results were expressed as mg gallic acid equivalents (GAE) per gram of dry matter (DM) of plant material (Jurinjak Tušek et al., 2016; Benković et al., 2017; Valinger et al., 2017).
Antioxidant activity (AOX) was determined using two methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method (Brand-Williams et al., 1995) and Ferric Reducing Antioxidant Power (FRAP) method (Benzie and Strain, 1996). All analyses were performed in duplicate and the results were expressed as mmol Trolox equivalents per gram of dry matter (DM) of plant material and as mmol $FeSO_4 \cdot 7H_2O$ equivalents per gram of dry matter (DM) of plant material, respectively (Benković et al., 2017).

Statistical analysis

Analysis of Variance (ANOVA) was applied to determine the influence of individual variables (five extraction parameters) simultaneously on output variables (total polyphenolic content, antioxidant activity) in the ethanol/water solvent extraction of bioactives originated from the industrial hemp, with significance level of $p<0.05$. The adequacy of the second-order polynomial model was evaluated by the coefficient of determination (R^2). Experimental design and statistical analysis were performed using software Statistica v.10.0 (StatSoft Inc., Tulsa, USA).

Results and discussion

Chemical characteristics of the prepared hemp extracts

In this work, parameters of the solid-liquid extraction were defined in order to ensure maximum values of the total polyphenolic content and antioxidant activity (determined by the DPPH and the FRAP methods) of the hemp extracts.

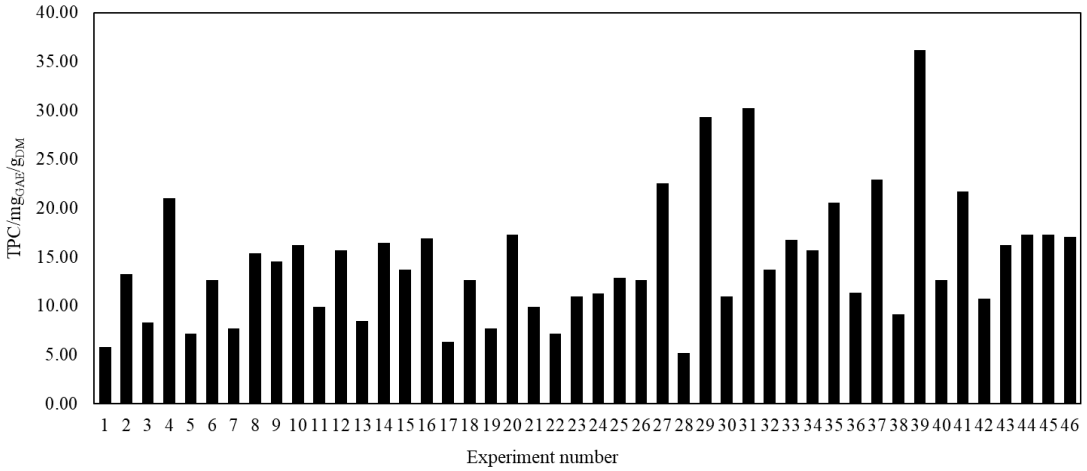

Fig. 1. The total polyphenolic content (TPC) at different experimental conditions

According to the results presented in Fig. 1, maximum TPC was extracted in exp. no. 39 (t = 25 min, T = 45 °C, 750 rpm, ethanol content = 25% and liquid to solid ratio = 50 mL/g); TPC = 36.160 mg_{GAE}/g_{DM} which is 4 fold higher compared to exp. no. 38 (t = 25 min, T = 45 °C, 750 rpm, ethanol content = 25% and liquid to solid ratio = 10 mL/g); TPC = 9.154 mg_{GAE}/g_{DM}. Experiments no. 38 and no. 39 differ only in the liquid to solid ratio and it can be concluded that higher liquid to solid ratio, the greater TPC amount will be extracted (Sifaoui et al., 2016). Minimum TPC value was obtained in exp. no. 28 (t = 5 min, T = 45 °C, 500 rpm, ethanol content = 25% and liquid to solid ratio = 10 mL/g); TPC = 5.218 mg_{GAE}/g_{DM}.
In the study of Drinić et al. (2018), water and ethanol content (30%, 50%, 70% and 90%) were used as a solvent in the extraction of two different samples of hemp (*Cannabis sativa* L.). It was determined that ethanol/water mixture (50%) was the best solvent for the extraction of phenolic compounds from both hemp samples (TPC = 17.05 mg GAE/g_{dw} and 9.25 mg GAE/g_{dw} for young and mature hemp, respectively). Regarding this work, higher TPC values were obtained in the experiments no. 4, 10, 12, 27, 41, 43 with ethanol content = 50%.

Cao et al. (2016) investigated optimum conditions of microwave-assisted extraction of total flavonoids (TF) from powders of hemp leaves. The influence of four factors (ethanol concentration: 40, 60, 80 %; liquid to solid ratio: 30:1, 35:1, 40:1 mL/g; extraction time: 10, 20, 30 min; temperature: 50, 60, 70 °C) for maximum recovery of TF yield was determined, using Box-Behnken design. The optimum microwave extraction conditions for obtain maximal yield of TF was as follows: solvent-to-solid ratio of 31.69 mL/g, extraction time of 25.14 min and extraction temperature of 69.96 °C. The predicted extraction yield of TF was 3.06%, which was in accordance with the experimental yield of 3.04 ± 0.62 %. Our study, together with the study of Cao et al. (2016), shows successful application of RSM methodology for modeling and optimization of extraction process biocompounds originating from hemp material.

Mkpenie et al. (2012) used organic solvents such as methanol, acetone and their 50% aqueous solutions for the extraction of *C. sativa* leaves. Extraction was performed on a shaker at room temperature for 2, 8 and 18 hours. According to the obtained results, different solvent extraction systems showed a wide range of polyphenols concentration: from 0.09 to 0.556 mg (GAE)/g_{dw}, depending on the solvent used. The highest TPC levels were achieved using methanol, followed by acetone, 50% methanol and 50% acetone. Compared to our work, higher TPC values were obtained using ethanol/water solvent, with shorter extraction time and the whole plant material was used for the extraction experiments.

Jokić et al. (2010) investigated the influence of the solvent, temperature and extraction time on the extraction yield of total polyphenols from milled soybeans variety IKA. The best extraction yield of total polyphenols was obtained using 50% aqueous ethanol solution at 50 °C after 60 min (TPC = 3.045 mg GAE/g_{DM}), compared to the extraction yield of total polyphenols obtained using water as a solvent (1.119 mg GAE/g_{DM}). The similar results were obtained in this work, in the experiments no. 26 (t = 25 min, T = 60 °C, 500 rpm, ethanol content = 0% and liquid to solid ratio = 30 mL/g); TPC = 12.633 mg_{GAE}/g_{DM} and no. 27 (t = 25 min, T = 60 °C, 500 rpm, ethanol content = 50% and liquid to solid ratio = 30 mL/g); TPC = 22.520 mg_{GAE}/g_{DM}. It can be concluded that the combination of water with organic solvent will ensure optimal conditions for polyphenols extraction from different plant materials (Rafiee et al., 2011; Dent et al., 2013).

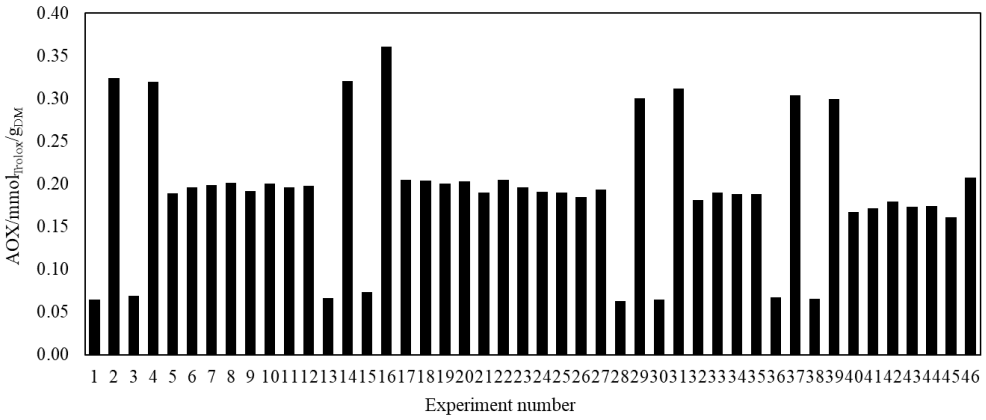

Fig. 2. The antioxidant activity (AOX) determined by the DPPH method, at different experimental conditions

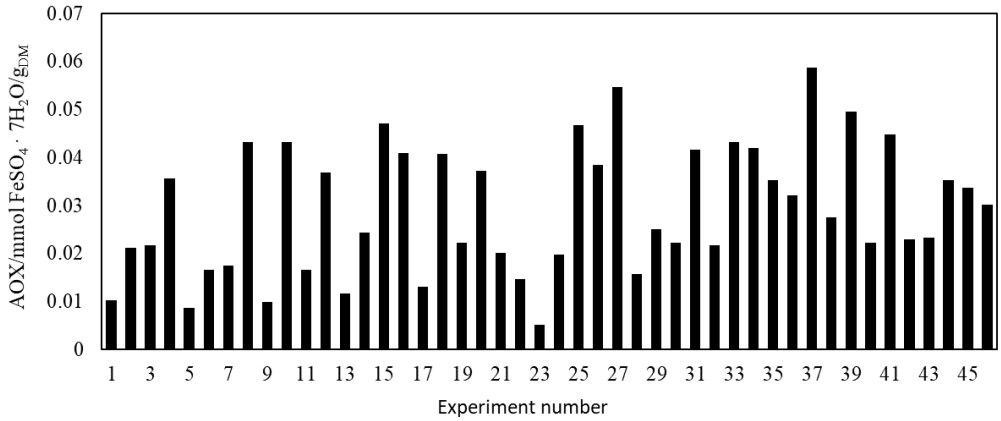

Fig. 3. The antioxidant activity (AOX) determined by the FRAP method, at different experimental conditions

Regarding AOX determined by the DPPH method (Fig. 2), the highest value was obtained in exp. no. 16 (t = 25 min, T = 60 °C, 500 rpm, ethanol content = 25% and liquid to solid ratio = 50 mL/g); DPPH = 0.361 $mmol_{Trolox}/g_{DM}$ while the lowest value was determined in experiment no. 28 (t = 5 min, T = 45 °C, 500 rpm, ethanol content = 25% and liquid to solid ratio = 10 mL/g); DPPH = 0.062 $mmol_{Trolox}/g_{DM}$. Regarding AOX determined by the FRAP method (Fig. 3) the highest value was obtained in exp. no. 37 (t = 25 min, T = 45 °C, 250 rpm, ethanol content = 25% and liquid to solid ratio = 50 mL/g); FRAP = 0.059 mmol $FeSO_4 \cdot 7H_2O/g_{DM}$ while the lowest value was obtained in exp. no. 9 (t = 25 min, T = 45 °C, 250 rpm, ethanol content = 0% and liquid to solid ratio = 30 mL/g); FRAP = 0.001 mmol $FeSO_4 \cdot 7H_2O/g_{DM}$.

In the study of Mkpenie et al. (2012), antioxidant activity was determined by reducing power assay and it was in the range from 0.202 to 0.866 mg/mL. Authors used methanol, acetone and their 50% aqueous solutions to perform polyphenols extraction from hemp leaves.

Drinić et al. (2018) expressed antioxidant activity in terms of EC_{50} values. These values were in the range from 0.1321 to 0.4353 mg/mL for young hemp and from 0.2055 to 0.7563 mg/mL for mature hemp. The value of EC_{50} is inversely related to its antioxidant activity and it presents the concentration of the test sample which neutralizes 50% of DPPH radicals. The lowest value of EC_{50} means the highest antioxidant activity. The highest antioxidant activity was obtained with 30% ethanol for young hemp (EC_{50} = 0.1321 mg/mL) and 50% ethanol for mature hemp (EC_{50} = 0.2055 mg/mL).

Compared to our results regarding the antioxidant activity with the results of the above mentioned authors, it can be concluded that different ethanol/water mixtures are better solvents for the extraction of polyphenols from industrial hemp.

Optimization of extraction conditions considering chemical characteristics of the hemp extracts

In this work, the effects of five factors (extraction temperature, liquid to solid ratio, extraction time, rotational speed and ethanol/water solvent ratio) on the total polyphenolic content (TPC) and antioxidant activity (AOX) (determined by the DPPH and the FRAP methods) of the hemp extracts were analysed. The results of optimization are presented in Fig. 4. Based on the obtained results, increment of the liquid to solid ratio up to 50 mL/g had significant effect on increase in TPC content and AOX determined by the DPPH and the FRAP methods. The higher the solvent to solid ratio, the higher the total amount of solids obtained, which is in accordance with literature data (Radojković et al., 2012; Sifaoui et al., 2016). In our work, maximum values of TPC, AOX (DPPH) and AOX (FRAP) were determined in the experiments with liquid to solid ratio 50 mL/g (exp. no. 39, 16 and 37, respectively) (Figs. 1-3). The extraction temperature is an important parameter in process optimization since high temperatures may lead to degradation of antioxidant compounds (Spigno et al., 2007; Radojković et al., 2012). In this work, the impact of temperature on the TPC, AOX (DPPH) and AOX (FRAP) was investigated in the range of 30 °C – 60 °C. TPC increased with the increase of temperature up to 45 °C after which further temperature increase did not cause significant changes in the TPC. Maximum TPC value was obtained at 45 °C (exp. no. 39) (Fig. 1). Temperature increase did not affect AOX determined by the DPPH method, but it influenced the AOX determined by the FRAP method. Similar values for AOX (FRAP) were obtained in exp. no. 27 at T = 60 °C (FRAP = 0.055 mmol $FeSO_4 \cdot 7H_2O/g_{DM}$), and exp. no. 37 at T = 45 °C (FRAP = 0.059 mmol $FeSO_4 \cdot 7H_2O/g_{DM}$), although these two experiments differed considering temperature, liquid to solid ratio, ethanol content and rotational speed (Fig. 3, Table 1). Regarding ethanol/water solvent ratio, increase in ethanol content from 0% to 50% had a significant effect on AOX (FRAP) while it did not influence AOX (DPPH) and it had a small influence on the TPC content. According to Rostango et al. (2004), it is necessary to add a certain amount of water to the extraction solvent to improve extraction of phenolics, although water content higher than 60% can result in reduction of the extraction yield of phenolic compounds. The impact of rotational speed on TPC and AOX was investigated in the range of 250 – 750 rpm. The increment of rotational speed did not influence on TPC and AOX (DPPH) while it influenced AOX (FRAP): in the range of 250 – 500 rpm values of AOX (FRAP) decreased, but in the range of 500 – 750 rpm AOX (FRAP) values increased. Extraction time did not affect AOX (DPPH) and TPC while it had a minor influence on AOX (FRAP): similar AOX determined by the FRAP method were obtained for 25 and 45 minutes (Fig. 3, Table 1).

Based on the results from Fig. 4, the optimum process conditions for the ethanol/water extraction of bioactives from the hemp, with regard to chemical properties were: t = 25 min, T = 45 °C, 500 rpm, ethanol content = 25% and liquid to solid ratio =

30 mL/g with values of the TPC = 13.27 mg_{GAE}/g_{DM}, AOX (DPPH) = 0.19 $mmol_{Trolox}/g_{DM}$, AOX (FRAP) = 0.02 mmol $FeSO_4 \cdot 7H_2O/g_{DM}$. Optimum experimental values for AOX (DPPH) and AOX (FRAP) were close to the RSM predicted values while optimum experimental value for TPC somehow differed from the RSM predicted TPC value. Model predicted values were: TPC = 20.69 mg_{GAE}/g_{DM}, AOX (DPPH) = 0.21 $mmol_{Trolox}/g_{DM}$, AOX (FRAP) = 0.03 mmol $FeSO_4 \cdot 7H_2O/g_{DM}$.

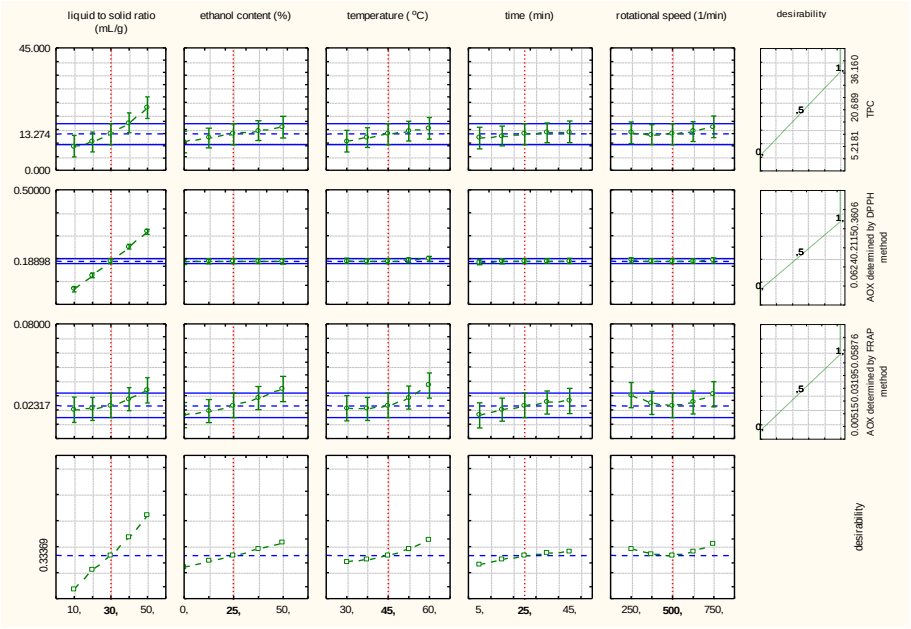

Fig. 4. Results of the optimization of the solid-liquid extraction of bioactives from industrial hemp with respect to chemical properties (TPC and AOX)

Analysis of variance (ANOVA)

Analysis of variance (ANOVA) was used to investigate the effect of process variables on the chemical characteristics of the hemp extracts. Regression parameters, together with determination coefficients, are presented in Table 2. The results indicated that linear parameters were positive for four investigated process variables, while the quadratic effects of independent variables demonstrated both positive and negative effects. The ANOVA for independent variables indicated that liquid to solid ratio was the most significant factor (p<0.05) affecting TPC, AOX (DPPH) and AOX (FRAP). The model indicated that liquid to solid ratio had positive linear effects on TPC and both antioxidant activities. The largest positive linear regression coefficient of the liquid to solid ratio was obtained for the TPC while the smallest value was obtained for AOX (FRAP). Ethanol content and temperature contributed significantly to the TPC and AOX (FRAP). On the other hand, these two parameters had no significant effect on AOX (DPPH). The extraction time did not significantly influence the chemical characteristics of the hemp extracts (p>0.05). The negative value of quadratic regression coefficient of rotational speed significantly influenced AOX (FRAP) (p<0.05) while this parameter had no effect on the TPC and AOX (DPPH). Considering the values of the linear regression coefficients, obtained for independent and dependent variables, liquid to solid ratio, ethanol content and extraction temperature were perhaps the most important factors contributing TPC extraction from the hemp using RSM methodology.

Based on the calculated determination coefficients it can be concluded that influence of process variables on AOX (FRAP) cannot be described well by the developed prediction models (R^2 = 0.540). On the other hand, determination coefficient calculated for AOX (DPPH) was higher than 0.9 (R^2 = 0.976), indicating a strong relationship between DPPH values and investigated process variables while in the case of TPC (R^2 = 0.603), there is a good relationship between the TPC and process variables.

Table 2. Regression coefficients and determination coefficients obtained by Box-Behnken experimental design for chemical properties of hemp extracts (bold values: $p<0.05$)

Analysed property	Parameter	Intercept ± standard error	Regression coefficients (L-linear, Q- quadratic) ± standard error	*P*	R^2	R^2adj.
TPC (mg_{GAE}/g_{DM})	Liquid to solid ratio (mL/g)	15.719 ± 1.763	**7.083 ± 1.164(L)** -1.342±0.788(Q)	**0.000(L)** 0.097(Q)	0.603	0.490
	Ethanol content (%)		**2.741±1.164(L)** 0.074±0.788(Q)	**0.024(L)** 0.926(Q)		
	Temperature (°C)		**2.3687±1.164(L)** 0.108±0.788(Q)	**0.049(L)** 0.892(Q)		
	Extraction time (min)		1.156±1.164(L) 0.138±0.788(Q)	0.327(L) 0.862(Q)		
	Rotational speed (rpm)		1.104±1.164(L) -0.812±0.788(Q)	0.349172(L) 0.310083(Q)		
DPPH ($mmol_{Trolox}$/gDM)	Liquid to solid ratio (mL/g)	0.193 ± 0.005	**0.125±0.003(L)** -0.001±0.002(Q)	**0.000(L)** 0.634(Q)	0.976	0.970
	Ethanol content (%)		0.001±0.003(L) 0.001±0.002(Q)	0.750(L) 0.525(Q)		
	Temperature (°C)		0.004±0.003(L) -0.003±0.002(Q)	0.247(L) 0.233(Q)		
	Extraction time (min)		0.003±0.003(L) 0.001±0.002(Q)	0.419(L) 0.707(Q)		
	Rotational speed (rpm)		-0.000±0.003(L) -0.001±0.002(Q)	0.899(L) 0.526(Q)		
FRAP ($mmol_{FeSO4\cdot 7H2O}/g_{DM}$)	Liquid to solid ratio (mL/g)	0.035 ± 0.004	**0.007±0.003(L)** -0.002±0.002(Q)	**0.012(L)** 0.293(Q)	0.540	0.409
	Ethanol content (%)		**0.009±0.003(L)** -0.001±002(Q)	**0.001(L)** 0.514(Q)		
	Temperature (°C)		**0.008±0.003(L)** -0.003±0.002(Q)	**0.004(L)** 0.098(Q)		
	Extraction time (min)		0.0050±0.003(L) 0.0010±0.002(Q)	0.059(L) 0.578(Q)		
	Rotational speed (rpm)		0.000±0.003(L) **-0.004±0.002(Q)**	0.914(L) **0.040(Q)**		

Conclusions

Optimization represents an essential tool in food engineering and biotechnology with the aim to yield a highly acceptable product. In this work, Response Surface Methodology, coupled with Box-Behnken design, was applied to determine the optimal conditions of the ethanol/water solvent extraction of bioactive components from industrial hemp investigating some influential parameters such as liquid to solid ratio, temperature, ethanol content, extraction time and rotational speed. The optimum TPC content and antioxidant activity (DPPH and FRAP) in the hemp extracts were achieved with ethanol/water ratio 25% at 45 °C for 25 min, with solvent to plant ratio 30 mL/g and rotational speed 500 rpm. Under optimized conditions, the obtained experimental values agreed with the predicted values. According to the ANOVA results, liquid to solid ratio significantly influenced TPC, AOX (DPPH) and AOX (FRAP), rotational speed had significant influence only on AOX (FRAP) while ethanol content and temperature contributed significantly to the TPC and AOX (FRAP). This research serves as the basis for further investigations on the optimization of extraction procedure of bioactive components and antioxidant activity from industrial hemp using response surface methodology approach.

Literature

Andre, C.M., Hausman, J., Guerriero, G. (2016): *Cannabis sativa*: The plant of the thousand and one molecules, *Front. Plant Sci.* 7, 1–17. https://doi.org/10.3389/fpls.2016.00019

Appendino, G., Chianese, G., Taglialatela-Scafati, O. (2011): Cannabinoids: Occurrence and medicinal chemistry,*Curr. Med. Chem.* 18, 1085–1099. https://doi.org/10.2174/092986711794940888

Benković, M., Belščak-Cvitanović, A., Jurina, T., Valinger, D., Jurinjak Tušek, A., Jakelić, M., Gajdoš Kljusurić, J. (2017): Effect of extraction conditions on extraction yield, total polyphenolic content and antioxidant capacity of yarrow (*Achilea millefolium* L.). In: Proceedings of the 16th Ružička days, Jukić, A. (ed.), Osijek, HR, pp. 155–162. https://doi.org/10.1016/j.indcrop.2016.07.015

Benzie, I.F., Strain, J.J. (1996): The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, *Anal. Biochem*. 239, 70–76. https://doi.org/10.1006/abio.1996.0292

Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S., Escaleira, L.A. (2008): Response surface methodology (RSM) as a tool for optimization in analytical chemistry, *Talanta* 76, 965–977. https://doi.org/10.1016/j.talanta.2008.05.019

Brand-Williams, W., Cuvelier, M.E., Berset, C. (1995): Use of free radical method to evaluate antioxidant activity, *Lebensm. Wiss. Technol*. 28, 25–30. https://doi.org/10.1016/s0023-6438(95)80008-5

Cao, J., Hao, L., Zhang, L., Xu, M., Ge, H., Kang, C., Yu, J., Wang, Z. (2016): Optimization of microwave-assisted extraction of total flavonoids from China-hemp leaves and evaluation of its antioxidant activities. In: International Conference on Applied Biotechnology, Liu, H., Song, C., Ram, A., (eds.), Springer, Singapore, pp. 555–567. https://doi.org/10.1007/978-981-10-4801-2_57

Dent, M., Dragović-Uzelac, V., Penić, M., Bosiljkov, T., Levaj, B. (2013): The effect of extraction solvents, temperature and time on the composition and mass fraction of polyphenols in dalmatian wild sage (*Salvia officinalis* L.) extracts, *Food Technol. Biotech.* 51, 84–91. https://doi.org/10.5562/cca3231

Drinić, Z., Vidović, S., Vladić, J., Koren, A., Kiprovski, B., Sikora V. (2018): Effect of extraction solvent on total polyphenols content and antioxidant activity of *Cannabis sativa* L. *Lekovite Sirovine* 38, 17–21. https://doi.org/10.5937/leksir1838017D

Hartsel, J.A., Eades, J., Hickory, B., Makriyannis, A. (2016): *Cannabis sativa* and Hemp. In: Nutraceuticals: Efficacy, Safety and Toxicity, Gupta, R.C. (ed.), Academic press, Elsevier, pp. 735–754. https://doi.org/10.1016/b978-0-12-802147-7.00053-x

Jokić, S., Velić, D., Bilić, M., Bucić-Kojić, A., Planinić, M., Tomas, S. (2010): Modelling of the process of solid-liquid extraction of total polyphenols from soybeans, *Czech J. Food Sci*. 28, 206–212. https://doi.org/10.1016/b978-0-12-802147-7.00053-x

Jurinjak Tušek, A., Benković, M., Belščak-Cvitanović, A., Valinger, D., Jurina, T., Gajdoš Kljusurić, J. (2016): Kinetics and thermodynamics of the solid-liquid extraction process of total polyphenols, antioxidants and extraction yield from *Asteraceae* plants, *Ind. Crop. Prod.* 91, 205–214. https://doi.org/10.1016/j.indcrop.2016.07.015

Jurinjak Tušek, A., Benković, M., Valinger D., Jurina, T., Belščak-Cvitanović, A., Gajdoš Kljusurić, J. (2018): Optimizing bioactive compounds extraction from different medicinal plants and prediction through nonlinear and linear models, *Ind. Crops Prod.* 126, 449–458. https://doi.org/10.1016/j.indcrop.2018.10.040

Kolodziejczyk, P., Ozimek, L., Kozłowska, J. (2012): The application of flax and hemp seeds in food, animal feed and cosmetics production, *Wood. Publish. Lim.* 329–366. https://doi.org/10.1533/9780857095510.2.329

Kostić, M.D., Joković, N.M., Stamenković, O.S., Rajković, K.M., Milić, P.S., Veljković, V.B. (2013): Optimization of hempseed oil extraction by n-hexane, *Ind. Crops Prod.* 48, 133–143. https://doi.org/10.1016/j.indcrop.2013.04.028

Liu, L., Shen, B.-J., Xie, D.-H., Cai, B.-C., Qin, K.-M., Cai, H. (2015): Optimization of ultrasound-assisted extraction of phenolic compounds from *Cimicifugae rhizoma* with response surface methodology, *Phcog. Mag.* 11, 682–689. https://doi.org/10.4103/0973-1296.165545

Majeed, M., Hussain, A.I., Chatha, S.A.S., Khosa, M.K.K., Kamal, M., Kamal, M.A., Liu, M. (2016): Optimization protocol for the extraction of antioxidant components from *Origanum vulgare* leaves using response surface methodology, Saudi J. Biol. Sci. 23, 389–396. https://doi.org/10.1016/j.sjbs.2015.04.010

Mkpenie, V.N., Essien, E.E., Udoh, I.I. (2012): Effect of extraction conditions on total polyphenol contents, antioxidant and antimicrobial activities of *Cannabis sativa* L., *EJEAFChe* 11, 300–907.

Pellati, F., Borgonetti, V., Brighenti, V., Biagi, M., Benvenuti, S., Corsi, L. (2018): *Cannabis sativa* L. and nonpsychoactive cannabinoids: their chemistry and role against oxidative stress, inflammation, and cancer, *Biomed. Res. Int.* 2018, 1–15. https://doi.org/10.1155/2018/1691428

Pollastro, F., Minassi, A., Fresu, L.G. (2018): Cannabis phenolics and their bioactivities, *Curr. Med. Chem.* 25, 1160–1185. https://doi.org/10.2174/0929867324666170810164636

Radojković, M., Zeković, Z., Jokić, S., Vidović, S., Lepojević, Ž., Milošević, S. (2012): Optimization of solid-liquid extraction of antioxidants from black mulberry leaves by response surface methodology, *Food Technol. Biotechnol.* 50, 167–176.

Rafiee, Z., Jafari, S.M., Alami, M., Khomeiri, M. (2011): Microwave-assisted extraction of phenolic compounds from olive leaves; a comparison with maceration, *J. Anim. Plant Sci*. 21, 738–745.

Rostango, M.A., Palma, M., Barroso, C.G. (2004): Pressurized liquid extraction of isoflavones from soybeans, *Anal. Chim. Acta* 522, 169–177. https://doi.org/10.1016/j.aca.2004.05.078

Sarasini, F., Fiore, V. (2018): A systematic literature review on less common natural fibres and their biocomposites. *J. Clean. Prod.* 195, 240 – 267.

Shahid-ul-Islam, Shahid, M., Mohammad, F. (2013): Perspectives for natural product based agents derived from industrial plants in textile applications - a review, *J. Clean. Prod.* 57, 2–18. https://doi.org/10.1016/j.jclepro.2013.06.004

Sifaoui, I., Chammem, N., Abderrabba, M., Mejri, M. (2016): Optimization of phenolic compounds extraction from olive leaves using experimental design methodology. *J. Mater. Environ. Sci.* 7, 1119–1127.

Silva, E.M., Rogez, H., Larondelle, Y. (2007): Optimization of extraction of phenolics from *Inga edulis* leaves using response surface methodology. *Sep. Purif. Technol.* 55, 381–387. https://doi.org/10.1016/j.seppur.2007.01.008

Singleton, V.L., Rossi, J.A. (1965): Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, *Am. J. Enol. Viticult.* 16, 144–158.

Spigno, G., Tramelli, L., De Faveri, D.M. (2007): Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics, *J. Food Eng.* 81, 200–208. https://doi.org/10.1016/j.jfoodeng.2006.10.021

Tchabo, W., Ma, Y., Kwaw, E., Xiao, L., Wu, M., Apaliya, M.T. (2018): Impact of extraction parameters and their optimization on the nutraceuticals and antioxidant properties of aqueous extract mulberry leaf, *Int. J. Food Prop.* 21, 717–732. https://doi.org/10.1080/10942912.2018.1446025

Thomas, B. F., ElSohly, M. A. (2015): The Analytical Chemistry of *Cannabis*: Quality Assessment, Assurance and Regulation of Medicinal Marijuana and Cannabinoid Preparations, Elsevier, Amsterdam, pp. 1–132.

Valinger, D., Jurina, T., Benković, M., Jurinjak Tušek, A., Belščak-Cvitanović, A., Jakelić, M., Gajdoš Kljusurić, J. (2017): Determination of correlations between electrical conductivity, dry matter, total polyphenolic content and antioxidant capacity of marigold extracts (*Calendula officinalis*). In: Proceedings of the 16th Ružička days, Jukić, A. (ed.), Osijek, HR, pp. 189–197. https://doi.org/10.1016/j.indcrop.2016.07.015

Wang, W.Y., Zhou, Z.H., Huang, N.J., Yun-lan, Tang, S.Y. (2014): Study on the extraction of active ingredients in bamboo leaves by enzymatic coupling high pulse electric field, *Food Ferment. Tech.* 4, 42–45.

Xu, C.-C., Wang, B., Pu, Y.-Q., Tao, J.-S., Zhang, T. (2017): Advances in extraction and analysis of phenolic compounds from plant materials, *Chin. J. Nat. Medicines* 15, 0721–0731. https://doi.org/10.1016/S1875-5364(17)30103-6