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Application of conventional and high-pressure extraction techniques for the isolation of bioactive compounds from the aerial part of hemp (*Cannabis sativa* L.) assortment Helena

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ABSTRACT

In this work, different extraction techniques (soxhlet extraction, hydrodistillation, subcritical water extraction and supercritical carbon dioxide extraction followed by conventional extraction) were employed for the isolation of bioactive compounds from the areal parts of industrial hemp (*Cannabis sativa* L.). The extraction process parameters, time and temperature for subcritical water extraction and pressure, temperature and time for supercritical carbon dioxide extraction, on the extraction yield and the content of bioactive compounds from hemp were examined. As the plant material after supercritical carbon dioxide still contains hydrophilic compounds, conventional extraction was used for isolation of these. The content of cannabidiol, the main cannabinoid present in hemp, in supercritical carbon dioxide extracts was between 71.84–163.11 mg/g, while in soxhlet extract it was much lower (64.40 mg/g). In comparison to these the significantly lower cannabidiol content was detected in subcritical water extracts, ranging from 0.0039 to 0.0183 mg/mL. Comparing all applied extraction techniques, supercritical carbon dioxide followed by conventional extraction was selected as the most valuable process for bioactive compounds isolation for hemp.

1. Introduction

Cannabis sativa L. (hemp or industrial hemp) is a plant from the genus *Cannabis* that is cultivated primarily for the fiber and seeds production. Hemp fiber and seeds are used in different industries such as paper, textile, cosmetics, food and other. Today there is a growing trend for the use of industrial hemp for pharmaceutical purposes. Hemp synthesizes about 500 compounds that belongs to the different classes such as cannabinoids (the most studied compounds), terpenes, hydrocarbons, nitrogen compounds, carbohydrates, flavonoids, fatty acids, non-cannabinoid phenols, simple alcohols, aldehydes, ketones, and others (Brenneisen, 2007). The main cannabinoid present in the industrial hemp is cannabidiol (CBD) (Brighenti et al., 2017). CBD is a valuable compound because it possesses various pharmacological activities such as antioxidant and anti-inflammatory (Atalay et al., 2020), neuroprotective (Hampson et al., 1998), anxiolytic (Schier et al., 2012), antiepileptic (Devinsky et al., 2016), antifungal and antibacterial (Klingeren and Ham, 1976; McPartland, 1984). Beside of CBD, many authors also reported that industrial hemp is a suitable source of polyphenols (Mkpenie et al., 2012; Frassinetti et al., 2018; Fathordoobady et al., 2019; Nagy et al., 2019). The hemp contains different phenol compounds such as phenolic acid (hydroxycinnamic acids, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid), lignanamides (cannabisin A, B, and C), phenolic amides (N-*trans*-caffeoyltyramine), flavonoids (flavonol, rutin, quercetin-3-glucoside, kaempferol-3-O-glucoside, quercetin, kaempferol), flavones (cannflavin A and B, luteolin-7-O-glucoside, apigenin-7-O-glucoside, luteolin, apigenin), flavanols (catechin, epicatechin), flavanone (naringenin) (Izzo et al., 2020). Phenolic compounds of hemp possess antiinflamatory, anticancer and neuroprotective properties (Andre et al., 2010). Another group of hemp compounds that has important properties for further applications are terpenes. Due to their lipophilic characteristics' terpenes possess a

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wide range of biological activities such are: anticancer, anxiolytic, immune stimulating, antiinflamatory, analgesic, memory skills improving and gastro protective activity (Andre et al., 2016). Health beneficial properties of hemp bioactive compounds can be utilized if these bioactives are efficiently isolated from the herbal material of adequate quality. Therefore, it is of importance to investigate their isolation process, meaning to evaluate and select the most appropriate extraction technique and the most appropriate process conditions. So far, extraction of these compounds has been reported by various conventional extraction techniques such as maceration with the different solvents (Romano and Hazekamp, 2014; Drinić et al., 2018), soxhlet extraction (Wianowska et al., 2015; Attard et al., 2018; Lewis-Bakker et al., 2019), as well as advanced extraction methods such as microwave assisted extraction (Drinić et al., 2019; Lewis-Bakker et al., 2019), ultrasound assisted extraction (Agarwal et al., 2018; Lewis-Bakker et al., 2019), supercritical carbon dioxide extraction (Da Porto et al., 2014; Rovetto and Aieta, 2017; Kitrytė et al., 2018; Lewis-Bakker et al., 2019), pressurized liquid extraction (Wianowska et al., 2015; Kitrytė et al., 2018), and enzyme-assisted extraction (Kitrytė et al., 2018). According to Baldino et al. (2020), pharmaceutical applications can require the substantial isolation of single cannabinoids for specific pharmaceutical targets. In these cases, the production of single cannabinoids from the extraction mixture should be achieved. When single high purity (99 % or more) compounds are required, it can be useful to apply chromatographic techniques at production scale (Baldino et al., 2020).

This study is focused on the investigation of the potential of two advanced extraction techniques, subcritical water extraction (SWE) and supercritical carbon dioxide (SC-CO_2) extraction, to be applied for the isolation of bioactive compounds from the areal parts of industrial hemp (*Cannabis sativa* L.) cultivar Helena. Many authors reported that SWE, recognized as the novel green extraction technology, was the efficient method for the isolation of the phenolic compounds from the different plant materials (Naffati et al., 2017; Ju et al., 2011). At the elevated temperature conditions, the SWE can be also applied for the isolation of compounds of lower polarity. To evaluate the potential of SWE for the isolation of hemp constituents the influence of SWE process parameters (time and temperature) on the extraction of phenolic, CBD and THC, from industrial hemp cultivar Helena was examined. To the best of our knowledge there is no available publications on the application of SWE for this purpose. Up to now SC-CO_2 extraction has been applied for the isolation of different bioactive compounds (cannabinoids, aromatic compounds) from different hemp parts. In the publications reported on the SC-CO_2 extraction of industrial hemp, the evaluation of the pressure, temperature and co-solvent addition effect on extraction efficiency was analyzed (Rovetto and Aieta, 2017; Brighenti et al., 2017; Kitrytė et al., 2018; Ribeiro Grijó et al., 2019). Study of Juárez et al. (2020) showed that the material left after SC-CO_2 extraction can be further applied for the isolation of highly polar constituents (Juárez et al., 2020); this can be considered as one of the SC-CO_2 advantages over extraction technique such as SWE. Therefore, in evaluation of SC-CO_2 extraction of areal hemp parts, beside the effect of pressure and temperature, investigation was also directed towards the effect of time, and for this the fractionation was performed and composition analysis of extracts fractions obtained in the different extraction time was analyzed. Further, after isolation of lipid and low polar compounds by SC-CO_2, herbal material left after extraction, as it still represents a source of hydrophilic compounds, was subjected to conventional ethanol extraction to investigate the possibility of its utilization for hemp phenolic constituent's isolation.

2. Material and methods

2.1. Plant material

The commercial crop of industrial hemp cultivar Helena was produced with technology recommended by Bócsa and Karus (1998) at experimental field in Bački Petrovac, Institute of Field and Vegetable Crops, Novi Sad, Serbia in 2017. The field sampling protocol was used according to Section 2, Appendix I of EU Regulation No. 796/200, procedure A for monoecious cultivars. The procedure includes cutting off top 30 cm of 700 randomly selected plant stems that contain at least one inflorescent, 20 days after the start of flowering of the crop (Callaway, 2008).

Sampled aerial parts (leaves, blossoms, small structural parts of the inflorescence and bracts) of the industrial hemp plants were air-dried at ambient temperature to a residual moisture (less than 12 %), afterward the stems and seeds were manually separated with test sieves (mesh 1.5 mm) and grind using a domestic blender, after which an average particle size of 0.4378 mm was determined by a sieve set (CISA Cedaceria Industrial, Spain). Such prepared plant material was used for supercritical fluid extraction, hydrodistillation, Soxhlet extraction and subcritical plant material.

2.2. Chemicals

Commercial carbon dioxide (Messer, Novi Sad, Serbia) with > 99.98 % (w/w) purity was used for laboratory scale SFE. Folin-Ciocalteu was purchased from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany). Both standard compounds, (±)-catechin and gallic acid, were purchased from Sigma (St. Louis, MO, USA). Analytical standards cannabidiol (CBD) and cannabinol (CBN), both with purity 99.95 % were purchased from Lipomed (Lipomed GmbH, Weil am Rhein, Germany). All other chemicals used in this study were of analytical reagent grade.

2.3. SC-CO_2 extraction followed by conventional extraction of phenolic constituents

The SC-CO_2 extraction was carried out on a laboratory scale high pressure extraction plant (HPEP, NOVA Swiss 565.0156, Effretikon, Switzerland) explained in detail by Vidović et al. (2011).

The plant material (40.0 g) was placed in an extractor vessel and the extraction process was carried out at different combinations of pressure (100, 200 and 300 bar) and temperature (40, 50 and 60 °C). Separator conditions were 15 bar and 23 °C. Extractions were conducted in duplicate. For each combination of parameters, 5 extracts were obtained: the extract obtained after extraction for 4 h, designated as total extract (TE), and fractions obtained after each hour of extraction (F1, F2, F3, F4). For observation of process kinetics, the extraction yield was measure after each 30 min until the total extraction time of 120 min and then after the extraction time of 180 and 240 min. The extracts were stored in the glass bottles at 4 °C until furthered analysis of extracts (chemical composition of aromatic constituents and the content of CBD and THC).

After SC-CO_2 extraction the exhausted plant material was collected and extracted by classical extraction (CE) at the room temperature for 24 h with 50 % ethanol as extraction solvent, applying solid/liquid ratio of 1:10. After extraction, obtained extracts were immediately filtered through the filter paper with pore size 4−12 μm (Schleicher & Schuell, Dassel, Germany) under vacuum (V-700, Buchi, Switzerland) and stored in the glass bottles in the freezer until the analysis of extraction yield (EY), the content of total phenols (TP) and total flavonoids (TF).

2.4. Subcritical water extraction (SWE)

SWE was performed in batch type high-pressure extractor Parr 4520 (Parr Instrument Company, USA). The extractor volume was 2 L, it was equipped with the continual mixing system, and with electrical heating system which can provide up to 350 °C working temperature. The plant material (15 g) was mixed with distillated water (150 mL) and extracted at different temperatures from 120 °C to 220 °C, with an increase of 20 °C. Extraction pressure of 30 bar (obtained by introduction of nitrogen) and extraction time (10 min) were constant. Based on the certain output parametric-qualitative characteristics of the obtained extracts (content

of CBD), the optimal temperature (140 °C) was selected for the investigation of the time impact, therefore the another set of extraction experiments were further performed at different extraction time (5, 10, 15, 30 and 40 min) at the constant temperature. After extraction, the extracts were immediately filtered through filter paper with an increased size of 4−12 μm (Schleicher & Schuell, Dassel, Germany) under vacuum (V-700, Buchi, Switzerland) and stored in a glass bottles in the freezer until further analysis of extraction yield (EY), the content of CBD and THC, content of total phenols (TP) and total flavonoids (TF).

2.5. Hydrodistillation

Hydrodistillation was performed for isolation of essential oil from aerial parts of industrial hemp using a Clevenger type apparatus according to the European Pharmacopeia (Ph. Eur. 8.0., 2013). The essential oil was collected and dried over anhydrous sodium sulphate. The essential oil yield, expressed as a percentage, was calculated on a moisture-free basis. Oil samples (20 μL) were dissolved in EtOH 96 % up to a total volume of 2 mL. Prepared samples of essential oil were stored in the freezer until further analysis of low polar and aromatic constituents by GC–MS.

2.6. Soxhlet extraction

Total lipids have been isolated by the application of the soxhlet method. In the applied method 10 g of industrial hemp was extracted by 100 ml of hexane using soxhlet apparatus. Extraction time was 6 h. After extraction, extraction solvent was evaporated under vacuum (V-700, Buchi, Switzerland). Obtained extract was stored in the glass bottle in a freezer until further analysis.

2.7. Content of CBD and THC

The content of CBD and THC in extracts obtained by SC-CO_2 extraction, SWE and soxhlet extraction of areal parts of industrial hemp were determined by GC–MS analysis. In the case of extract obtained by SWE absolute methanol (2.5 mL) was added to 0.5 ml of extract, shaken and after that centrifuged at 10,000 rpm for 5 min. The supernatant was transferred to GC vial. Extracts obtained by SC-CO_2 extraction and soxhlet extraction were dissolved also in the methanol and transferred to GC vial. Decarboxylation step of acidic form of CBD and THC was achieved in the GC–MS inlet at temperature of 280 °C. Analysis of cannabinoids was performed on Agilent 6890 N GC equipped with mass spectrum (MS) detector Agilent 5975B. The separation was performed on a fused silica capillary column (HP-5MS, 30 m ×0.25 mm i.d., and 0.25 μm film thickness). Helium was used as carrier gas at a constant flow of 1 mL/min. The temperature program was as follows: initial temperature of 200 °C was held for 2 min, then increased to 240 °C at a rate of 10 °C/min, and hold for 10 min. The injector and detector temperatures were set at 280 and 230 °C, respectively. The injected sample volume was 1.5 μL and split ratio was 1:20. Individual analytical standards for cannabidiol (CBD) and cannabinol (CBN) were used for calibration. Quantitation of THC was performed with CBN analytical standard in accordance with the method given by Poortman-van der Meer and Huizer (1999).

2.8. GC/MS analysis of low polar constituents in essential oil and extracts obtained by SC-CO_2 extraction and soxhlet extraction

The chemical composition of the essential oil, and extracts obtained by SC-CO_2 extraction and soxhlet extraction were analyzed using GC–MS technique. GC–MS analyses were performed on a Shimadzu GCMS-QP2010 ultra mass spectrometer fitted with a flame ionic detector and coupled with a GC2010 gas chromatograph. The InertCap5 capillary column (60.0 m ×0.25 mm ×0.25 μm) was used for separation. Helium (He), at a split ratio of 1:5 and a linear velocity of 35.2 cm/s was used as carrier gas. Initially, the oven temperature was 60 °C, which was held for 4 min, then increased to 280 °C at a rate of 4 °C/min, and held for 10 min. The injector and detector temperatures were adjusted at 250 °C and 300 °C, respectively. The ion source temperature was 200 °C. The identification of the constituents was performed by comparing their mass spectra and retention indices (RIs) with those obtained from authentic sample sand/or listed in the NIST/Wiley mass-spectra libraries, using different types of searches (PBM/NIST/AMDIS) and available literature data (Hochmuth and Hamburg, 2006; Adams, 2007).

2.9. Total phenols content

Total phenols content (TP) in the liquid extracts, obtained by SWE and CE, was determined by the Folin-Ciocalte spectrophotometric procedure (Kähkönen et al., 1999; Singleton and Rossi, 1965). The absorbance was measured on a spectrophotometer (6300 Spectrophotometer, Jenway, UK) at 750 nm. The TP was determined based on the calibration curve of standard gallic acid solution. The TP has been expressed as mg of gallic acid equivalent per mL of liquid extract (mg GAE/mL). All experiments were replicated three times and results are expressed as mean values.

2.10. Total flavonoids content

Total flavonoids content (TF) was determined in the liquid extracts, obtained by SWE and CE, according to the procedure descripted by Harborne (1999). The sample absorption was measured on a spectrophotometer (6300 Spectrophotometer, Jenway, UK) at 510 nm. The TF was determined based on the calibration curve of standard catechin solution. The TF was expressed as mg of catechin equivalent per mL of liquid extract (mg CE/mL). All experiments were replicated three times and results were expressed as mean values.

2.11. Statistical analysis

All experiments were performed in triplicates determinations. Results were presented as mean value ± standard deviation. One-way ANOVA was conducted to test the individual factors influence on observed property and Duncan post hoc test was used for differences between the mean values detection. Significant levels were considered at $p \leq 0.05$ (STATISTICA v.7.0.3). Statistical analysis was per-formed using the MS Office Excel v. 2010.

3. Results and discussion

3.1. Chemical profile of hemp extract obtained by soxhlet extraction

For characterization of the chemical profile of investigated industrial hemp cultivar Helena two extraction methods have been applied to isolate the low polar compounds present, soxhlet method and hydrodistillation. Both methods have been previously used for this kind of evaluation of different hemp parts. Thus, Lewis-Bakker et al. (2019) investigated different extraction techniques (soxhlet extraction, SC-CO_2 extraction, ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE)) of resin from flowers of 3 different varieties of *C. sativa*. According to this study soxhlet extraction with the ethanol as extraction solvent resulted in the total extraction yielded from 21 to 31 % (m/m), while in some extracts the cannabinoids content ranged from 2.0–9.2% (m/m) for THCA, 2.5–72.5% (m/m) for CBDA, 7.5–18.3% (m/m) for THC and 7.8–15.4% (m/m) for CBD. In this study, compared to the other investigated extraction techniques, soxhlet extraction was marked as the most efficient extraction technique for extraction cannabinoids in their neutral form. Attard et al. (2018) investigated the soxhlet extraction by heptane as extraction solvent and SC-CO_2 extraction of hemp dust residue. The authors reported that hemp dust extracts were rich in high-value lipophilic compounds such as fatty

acids, triterpenes, polycosan, aldehydes, hydrocarbons, sterol and cannabinoids. CBD content was 5832 μg/g. According to Attard et al. (2018) soxhlet extraction yield achieved 9.85 %, while cannabinoid content was 64.40 mg/g for CBD and 2.90 mg/g for THC, but the results showed SFE was more efficient with the respect to cannabinoid content.

Chemical composition of extract obtained from investigated sample (areal part of industrial hemp cultivar Helena) by application of soxhlet method was determined by GS/MS and relative percentages are present in the Table 1 and in Fig. 1. In the analyzed extract total 5 compounds were identified with CBD as the major (84.42 %). Attard et al. (2018) reported that the most abundant compounds in soxhlet hemp dust extracts were fatty acids except for one extract (obtained from dust collection from rotary screen of de duster) which contained the CBD as the major compound. The fatty acids present in the extracts obtained in Attard et al. (2018) were palmitoleic acid, oleic acid, linoleic acid and linolenic acid. In the investigated extract obtained from areal hemp part cultivar Helena palmitic acid was the only fatty acid present in hemp extract obtained by soxhlet method. Nonacosane was predominant hydrocarbon in the hemp dust residue extracts obtained in study by Attard et al. (2018), while the case of hemp cultivar Helena extracts the dominant hydrocarbon was hentriacontane.

3.2. Chemical profile of hemp essential oil

The investigated industrial hemp essential oil yield was 0.08 % and its chemical composition is presented in the Table 2 and on Fig. 2. A total of 44 identified compounds belong to the different classes such as monoterpenes, sesquiterpenes, diterpenes, cannabinoids and other. The most dominant compounds were sesquiterpenes with the relative percent greater than 90. Content of sesquiterpene hydrocarbons were 65.57 %, while content of oxygenated sesquiterpenes were 21.73 %. Cannabinoids were present in the relative percent of 7.6, while percent of monoterpene hydrocarbons and oxygenated monoterpenes were 3.29 and 0.40, respectively. The most abundant sesquiterpene was *trans*-caryophyllene followed by α-humulene, caryophyllene oxide, *trans*-*β*-farnesene, humulene epoxide II, *trans*-14-hydroxy-9-epi-caryophyllene, *trans*-*α*-bergamotene, caryophylla-4(12),8(13)-dien-5-α-ol, caryophylla-4(12),8(13)-dien5-β-ol, *β*-selinene. The major monoterpene compound identified was *α*-pinene (1.22 %). The main cannabinoid compounds detected were cannabidiol and cannabichromene. Relative percentage of CBD was app. 20 times higher than the cannabichromene.

Naz et al. (2017) investigated the isolation of essential oil from *C. sativa* and *C. indica* by different extraction methods – hydrodistillation, steam distillation, and SC-CO_2 extraction. Essential oil yield for *C. indica* was in range from 0.024 to 0.035 % for hydrodistillation, from 0.017 to 0.032 % for steam distillation, and from 0.031 to 0.039 % for SFE, while for *C. sativa* essential oil yield was from 0.021 to 0.029 %, from 0.015 to 0.020 %, and from 0.022 to 0.031 %, respectively. The essential oil content obtained in our study was several times higher, which indicates that cultivar Helena is a cultivar of a good potential. Naz et al. (2017) also concluded that the chemical composition of hemp essential oils did not differ significant depending on the extraction method applied. The most abundant compound in hemp essential oil were sesquiterpenes, which is in accordance with the results obtained in our study. Comparing the composition of essential oil of *C. sativa* and *C. indica*, it was found that *C. sativa* essential oil contained higher content of sesquiterpenes, while *C. indica* essential oil contained higher content of monoterpenes.

Mediavilla and Steinemann (1997) examined the chemical composition of essential oil of different sample of *C. sativa* (15 chemotypes) and *C. indica* (4 chemotypes). In the Mediavilla and Steinemann study detected essential oil yield was app. 0.13 %, slightly higher than the yield obtained from the areal parts of Helena cultivar (0.08 %). According to Mediavilla and Steinemann (1997), in all tested chemotypes, the most common compounds were monoterpenes with the percent from 47.90–92.10, while the sesquiterpenes content were from 5.20–48.60%. The major compound was myrcene (29.40–62.00%) followed by *trans*-caryophyllene (3.80–37.50%), *α*-terpinolene (0.40–23.80%), *α*-pinene (2.30–21.00%), and *trans*-ocimene (0.30–10.20%). The different chemical composition of obtained essential oils indicate that the chemical composition depends on the influence of the environment. The content of THC was 0.02 % for *C. sativa* cv. Fedora and 0.08 % for *C. indica* cv. Swismix, while CBD content was 0.25 % for *C. sativa* cv. Fedora and 0.04 % for *C. indica* cv. Swismix. The content of *trans*--caryophillene in essential oil obtained in study by Mediavilla and Steinemann (1997) was in accordance with its content obtained in this study, while CBD content was almost 30 times higher in the essential oil obtained in this study compared to the results present by the study of Mediavilla and Steinemann (1997) which indicates the high potential of industrial hemp of investigated cultivar Helena.

Zengin et al. (2018) examined the yield and chemical composition of sample of hemp essential oil collected in the different time period. Samples were collected per month, from September to October. The essential oil yield ranged from 0.19 to 0.31 % and was the highest in the sample collected on the beginning of September, while the lowest was in the sample collected on the beginning of October. The most common compounds were sesquiterpenes: *trans*-caryophyllene (28 %), caryophyllene oxide (15 %), *α*-humulene (13 %), *α*- and *β*-seline (7%) and *trans*-*α*-bergamotene (4%). The major monoterpene compounds were *α*- and *β*-pinene (11 %), myrcene (11 %), *α*-terpinolene (6%) and D-limonene (2%). Comparing the results obtained in our study for hydrodistillation of hemp cultivar Helena with the result obtained by Zengin et al. (2018) for hydrodistillation of hemp collected on the beginning of September, yield of essential oil was higher in the study of Zengin et al. (2018), while chemical composition was similar.

Much higher number of compounds was present in the hemp essential oil (44 identified) in comparison to the soxhlet extract where only 5 were identified. But in the soxhlet extract the content of CBD was dominant (84.42 %), while in the essential oil it was present in much lower percentage, just 7.6 %. Long lasting high temperature applied in the soxhlet extraction is the main reason of the absence of aromatic constituents in these extracts, but as the results are showing it is more efficient technique for the cannabinoids isolation.

3.3. SC-CO_2 extraction of hemp low polar constituents followed by the conventional extraction of phenolic compounds

According to the literature SC-CO_2 extraction was used for the extraction hemp seed oil (Da Porto et al., 2012; Aladić et al., 2015; Devi and Khanam, 2019), cannabinoids (Rovetto and Aieta, 2017; Brighenti et al., 2017; Kitrytė et al., 2018; Ribeiro Grijó et al., 2019) and aromatic compounds (Da Porto et al., 2014; Naz et al., 2017) from *C. sativa*. Published data, especially those considering isolation of cannabionids (Perrotin-Brunel et al., 2010), are showing that solubility of hemp cannabinoids in SC-CO_2 extraction increase with increasing the pressure, while increasing in the temperature results the increase in the solubility of CBG and THC and decrease in the solubility of CBD and CBN. Decrease in CBD and CBN solubility with increase the temperature is explained due to the melting point of these two cannabinoids which is close to the highest examined temperature. In line to this in this study

Table 1
Chemical composition of Soxhlet hemp extract.

	Compound	RI[a]	Relative percent [%]
1.	Caryophyllene oxide	1523.6	4.08
2.	Palmitic acid	1832.2	2.32
3.	Cannabidol	2248.5	84.42
4.	Hentriacontane	2647.7	7.41
5.	Nonacosane	2891.9	1.76

[a] RI, retention indices as determined on HP-5 column using homologous series of C8-C30 alkanes.

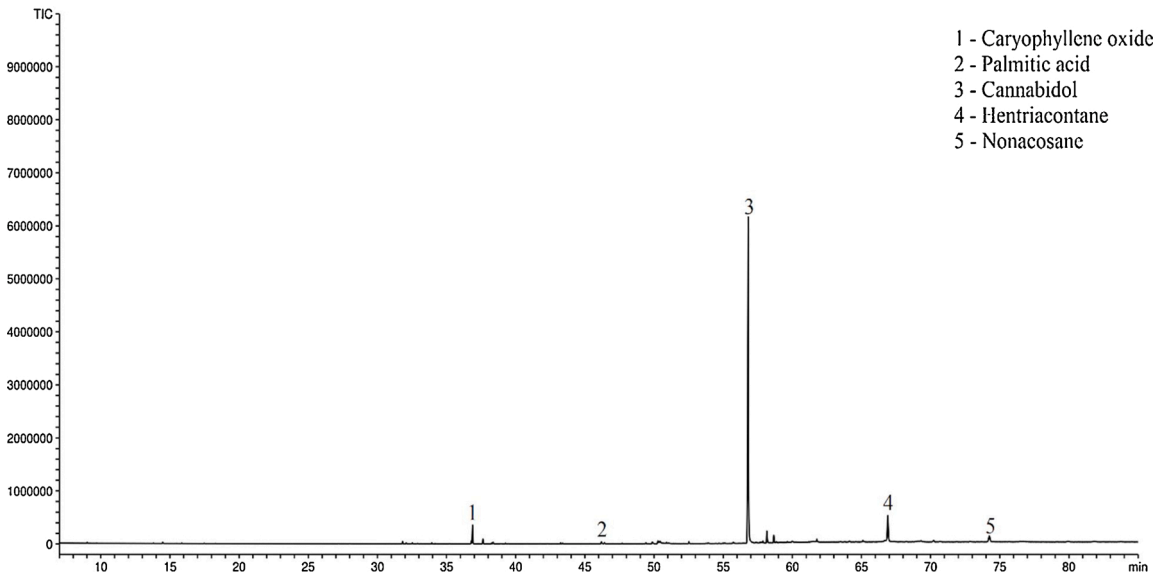

Fig. 1. GC/MS chromatogram of extract obtained from areal hemp part (assortment Helena) by application of soxhlet extraction.

the influence of different extraction parameters (pressure and temperature) on the extraction yield and the composition of industrial hemp cv. Helena extracts obtained by application of SC-CO_2 extraction was investigated. SC-CO_2 extraction was performed at the pressure of 100, 200 and 300 bar and temperature 40, 50, and 60 °C.

Achieved extraction yield ranged between 0.14 ± 0.04 and 7.05 ± 0.08 % (Table 3). Increasing the pressure at constant temperature, lead to the increase of the yield in all cases. The temperature showed different influence at the different pressure applied. At the constant pressure of 100 bar increased temperature showed negative influence on the extraction yield; at the pressure of 200 bar temperature impact was insignificant, while increasing the temperature at the pressure of 300 bar led to the significant increase of the extraction yield (from 5.18 ± 0.02 % (40 °C) to 7.05 ± 0.08 % (60 °C)). Increase of the extraction yield with the increase of the temperature and pressure is probably due to the increasing solvation power of SC-CO_2. Same was previously observed for the extraction of hemp seed oil (Aladić et al., 2015) and for the extraction of cannabinoids (Kitrytė et al., 2018; Attard et al., 2018), while temperature showed different influence. Some authors reported the decrease in the yield with increasing the temperature (Aladić et al., 2015; Gallo-Molina et al., 2019), while other reported increase in the yield with increasing the temperature (Kitrytė et al., 2018; Attard et al., 2018). In the case of hemp, the achieved SC-CO_2 extraction yield is highly dependent on the characteristics of the used plant material, i.e., variety, locality and growing conditions of the crop as well as the part of the plant used for extraction (Rovetto and Aieta, 2017). Attard et al. (2018) reported that SFE yield of hemp dust residue was between 0.12 and 1.57 %, while Rovetto and Aieta (2017) reported much higher SFE yields achieved in the extraction of hemp leaves and buds (from 7.40 to 18.50 %). Yield achieved in this study are in accordance with the literature data.

3.3.1. *Chemical profile of hemp total extract obtained by SFE*

The qualitative profile and the chemical constituents present in the hemp TE (extracts obtained after 4 h of extraction) are obtained using GC/MS and are presented in the Table 4 and on the Fig. 3. In total, 22 compounds have been identified belonging to the different classes of compounds such as monoterpenes, sesquiterpenes, diterpenes, triterpenes, cannabinoids, fatty acids, hydrocarbons and vitamins. The most abundant compounds in all total extracts were cannabinoids with the relative percent ranged from 65.48, for extract obtained at 100 bar and 50 °C, to 81.95, for extract obtained at 200 bar and 40 °C. The cannabinoid compound with the highest relative percentage from 62.44–78.26 as CBD. Other present cannabinoids were THC, p-heptyl acetophenone, and CBG with the relative percentage between 1.40 and 1.95, 0.92 and 1.41, and 0.92 and 1.41, respectively. CBC was present only in the extracts obtained at a pressure of 100 bar and at the extraction temperatures of 40 and 50 °C with the low relative percentage of 0.33 and 0.32, respectively. The content of sesquiterpenes hydrocarbons ranged from 7.91 to 23.92 %. The highest sesquiterpenes content was detected in the extract obtained at the pressure of 200 bar and temperature of 50 °C. The most dominant sesquiterpene was trans-caryophyllene following by α-humulene, caryophyllene oxide, cis-β-farnesene, trans-α-bergamotene. Monoterpenes, α-thujene and β-pinene with a low relative percentage 0.26 and 0.29, respectively, were present only in the extract obtained at temperature of 60 °C and pressure of 300 bar. The only diterpene present in the extracts was phytol, with a content from 0.36 to 0.72 %, while only triterpene present in the extracts was squalane, with a content from 0.88 to 1.54 %. The highest phytol and squalane content were determined in the extract obtained when extraction was performed at the pressure of 100 bar and temperature of 50 and 40 °C, respectively. Presence of terpenes can be significant in the case of cannabinoid extraction, namely as Russo (2011) was reported even a small amount of terpene significantly affects the activity of cannabinoids. Among fatty acid, palmitic acid was the only present compound, with the relative percentage between 1.30 and 4.40. α-tocopherol was present only in the extracts obtained at 100 bar, in very low percentages less than 1. Pentacosan was the major hydrocarbon and its relative percentage in the extracts ranged from 3.37 to 6.91.

Of the cannabinoids identified in SC-CO_2 extracts, the essential oil contains only three, CBD, CBC and, p-heptylacetophenone, while soxhlet extract contains only CBD. CBD content were similar in soxhlet and SC-CO_2 extracts, while in essential oil CBD content were app. 10x lower. CBC were present in same relative percentage in SC-CO_2 extracts and essential oil. The SC-CO_2 extraction was more efficient in the isolation of cannabinoids of different structure, with similar efficiency for CBD extraction as soxhlet method, and these facts can refer to the higher potential of this extraction technique. But, in the case of SC-CO_2 extraction THC was detected in extracts obtained by this technique while it was not the case with essential oil or extract produced by soxhlet method.

Generally, the number of sesquiterpene compounds detected in the essential oil was much higher in comparison to the other two extraction

Table 2
Chemical composition of hemp essential oil.

	Compound	RI[a]	Relative percent [%]
1.	*α*-pinene	926.1	1.22
2.	sabinene	966.7	0.44
3.	*β*-pinene	975.1	0.70
4.	*α*-phellandrene	996.8	0.08
5.	limonene	1013.6	0.40
6.	1,4-cineole	1016.6	0.17
7.	*cis*-*β*-ocimene	1028.2	0.15
8.	terpinolene	1068.3	0.31
9.	linalool	1075.8	0.08
10.	endo-fenchol	1093.2	0.08
11.	*α*-terpineol	1162.8	0.07
12.	*α*-ylangene	1329.8	0.08
13.	cyclosativene	1363.8	0.58
14.	*cis*-*α*-bergamotene	1367.0	0.36
15.	*α*-santalene	1372.8	0.33
16.	*trans*-caryophyllene	1379.1	38.30
17.	*trans*-*α*-bergamotene	1383.8	2.46
18.	geranyl acetone	1391.5	0.16
19.	*trans*-*β*-farnesene	1396.0	3.23
20.	α-humulene	1407.9	12.04
21.	allo-aromadendrene	1413.5	1.04
22.	*trans*-*β*-ionone	1427.4	0.48
23.	*α*-guaiene	1432.2	0.66
24.	*β*-selinene	1436.6	2.08
25.	*α*-selinene	1443.6	1.45
26.	*β*-bisabolene	1446.7	0.62
27.	*β*-sesquiphellandrene	1461.3	0.73
28.	*γ*-himachalene	1478.4	1.60
29.	trans-nerolidol	1492.0	1.45
30.	caryophyllene oxide	1525.0	6.73
31.	humulene epoxide II	1547.6	2.60
32.	selina-6-en-4-ol	1554.3	1.50
33.	Caryophylla-4(12),8(13)-dien-5-*α*-ol	1570.3	2.20
34.	Caryophylla-4(12),8(13)-dien5-*β*-ol	1586.6	2.13
35.	*trans*-14-hydroxy-9-epi-caryophyllene	1598.0	2.52
36.	epi-*α*-bisabolol	1602.8	1.18
37.	eudesm-7(11)-en-4-ol	1623.3	0.69
38.	nootkatone	1719.9	0.09
39.	hexahydrofarnesyl acetone	1733.8	0.38
40.	5-*trans*-9-*trans*-farnesyl acetone	1799.9	0.14
41.	phytol	1967.6	0.75
42.	*p*-heptylacetophenone	2070.4	0.14
43.	cannabichromene	2126.1	0.32
44.	cannabidiol	2247.0	7.28
Monoterpene hydrocarbons			3.29
Oxygenated monoterpenes			0.40
Sesquiterpene hydrocarbons			65.57
Oxygenated sesquiterpenes			21.73
Diterpenes			1.27
Cannabinoids			7.60
Other			0.14

[a] RI, retention indices as determined on HP-5 column using homologous series of C8-C30 alkanes.

technique. Relative percent of sesquiterpenes in SC-CO_2 extracts were higher than in extract obtained by soxhlet extraction where the only obtained sesquiterpene was caryophyllene oxide. Comparing with essential oil, sesquiterpenes content in SC-CO_2 extracts were lower but with the same dominant compound. Dominant sesquiterpene compound was trans caryophyllene. In the most extracts the α humulene and caryophyllene oxide where also present in the significant amounts. The relative percentage of all three was higher from app. 2 to 4 times in the essential oils. Phytol content was comparable in essential oil and SC-CO_2 extract, while in soxhlet extract it was not identified. Squalane as well as α-tocopherol were present only in SC-CO_2 extracts, as this technique is appropriate for their isolation. Palmitic acid was present in SC-CO_2 and soxhlet extracts with comparable content.

3.3.2. *Content of CBD and THC in extracts obtained by SC-CO_2 extraction*

The content of CBD and THC in TE ranged from 71.84–163.11 mg/g and from 3.66 to 6.58 mg/g, respectively (Table 5). The highest content of cannabinoids was obtained at the pressure of 100 bar and at the temperature of 40 °C. The lowest content of CBD was obtained at the same pressure, but at the temperature of 60 °C, while the lowest content of THC was obtained at the pressure of 200 bar and at the temperature of 50 °C. In general, the increase in the temperature, at all investigated values of pressures, had a negative effect on the cannabinoid isolation, except for THC isolation at 300 bar where the increase in temperature had positive influence. Similarly, the increase in the pressure leads to decrease in the content of cannabinoids in the extracts. Although the highest extraction yield was obtained at the highest value of the tested pressure and temperature, the cannabinoid content in the extract obtained under these conditions was not the highest. This is a possible consequence of increasing the solvation power of supercritical carbon dioxide by increasing the pressure. Increasing the solvation power of supercritical carbon dioxide reduces its selectivity, which leads to the extraction of a large number of other different compounds (Reverchon and De Marco, 2006). Similar to this, the increasing the yield and decreasing the cannabinoids content in SC-CO_2 extract of industrial hemp leaves and inflorescences with increasing the pressure was reported by Rovetto and Aieta (2017). Kitrytė et al. (2018) reported that the highest cannabinoid content in SFE of threshing residues of *C. sativa* cultivar 'Beniko' (mixture of leaves, floral bracts, flower fragments and immature seeds) was obtained in the extract produced at the lowest tested pressure (in the tested pressure range from 100 to 500 bar) and temperature (in the tested temperature range from 35 to 75 °C). Although the lowest investigated pressure and temperature were the most appropriate for the preparation of extract with the highest cannabinoid contents, the highest pressure and temperature provided generally the highest isolation of cannabinoids from the plant material (Table 5). In the recent review Baldino et al. (2020) gave the conclusion on the extraction of cannabionids with increased pressure in the process applying SC- CO_2. Namely, according to these authors, high levels of unwanted co-extracts are generally obtained when higher pressures are used to increase cannabinoids solubility in SC-CO_2. In particular, the larger extraction yield measured by increasing the operative pressure, corresponds to a low process selectivity of cannabinoids and not to an increase of their yield, since the coextraction of many undesired compounds is favored (Baldino et al., 2020).

The effect of temperature on the yield of isolated cannabinoids from the plant material at a pressure of 100 bar was the same as the effect of temperature on their content in the extracts and it decreases with the increasing temperature, while in the case of extraction at a pressure of 200 and 300 bar, the yield of isolated cannabinoids decreases with increasing the temperature up to 50 °C, while with further increase up to 60 °C it slightly increases. Increasing the pressure increases the yield of isolated cannabinoids. The mass of isolated CBD per g of plant material, for a defined range of extraction temperature, during extraction at a pressure of 100 bar ranged from 0.1782 to 2.2411 mg, at a pressure of 200 bar from 3.3525 to 6.4678 mg and at a pressure from 300 bar from 4.4917 to 7.7582 mg.

The influence of the extraction time on the content of CBD and THC in the hemp extracts was investigated by fractionation SC-CO_2. The obtained results indicate that over 70 % of cannabinoids were extracted in the first 120 min of extraction (Table 5). At the constant pressure of 100 bar in the first 120 min percentage of isolated cannabinoids were 75.04 for CBD and 71.50 for THC at temperature 40 °C, 81.20 for CBD and 74.29 for THC at temperature 50 °C, and 96.57 for CBD and 96.14 for THC at temperature 60 °C. At the constant pressure of 200 bar in the first 120 min of extraction the percent of isolated CBD and THC was 80.38 and 82.39 at a temperature of 40 °C, 77.03 and 80.25 at a temperature of 50 °C, and 76.27 and 81.59 at a temperature of 60 °C, respectively. Under the pressure of 300 bar and temperature of 40 °C, 50 °C and 60 °C 83.16 %, 89.03 % and 88.17 % of CBD and 85.94 %, 90.75 % and 90.63 % of THC are extracted in the first 120 min, respectively. The percentage of isolated CBD at different pressures and temperatures after each hour of extraction is present in Fig. 1. The temperature had

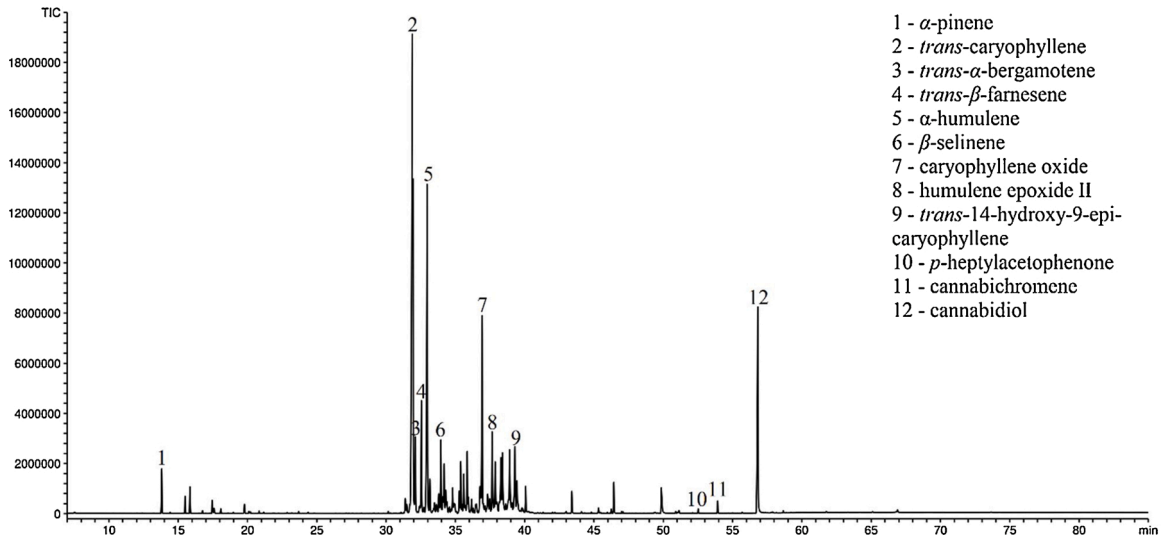

Fig. 2. GC/MS chromatogram of areal hemp part (assortment Helena) essential oil.

Table 3
Influence of SC-CO_2 extraction parameters (pressure and temperature) on extraction yield.

Extraction pressure (bar)	Extraction time (min)	Extraction temperature (°C)		
		40	50	60
100	30	0.32 ± 0.01	0.14 ± 0.04	0.19 ± 0.03
	60	0.61 ± 0.13	0.33 ± 0.06	0.22 ± 0.01
	90	0.94 ± 0.07	0.49 ± 0.02	0.27 ± 0.00
	120	1.15 ± 0.18	0.59 ± 0.01	0.30 ± 0.01
	180	1.45 ± 0.15	0.75 ± 0.01	0.35 ± 0.04
	240	1.68 ± 0.11	0.83 ± 0.05	0.36 ± 0.03
200	30	1.20 ± 0.10	1.29 ± 0.06	1.01 ± 0.08
	60	2.39 ± 0.03	2.18 ± 0.07	1.81 ± 0.12
	90	3.07 ± 0.09	2.98 ± 0.03	2.75 ± 0.04
	120	3.51 ± 0.21	3.52 ± 0.08	3.28 ± 0.02
	180	4.39 ± 0.22	4.39 ± 0.24	4.12 ± 0.03
	240	4.87 ± 0.21	4.97 ± 0.25	4.97 ± 0.01
300	30	1.37 ± 0.12	1.67 ± 0.04	2.17 ± 0.10
	60	2.47 ± 0.01	3.15 ± 0.07	3.65 ± 0.06
	90	3.45 ± 0.01	4.02 ± 0.15	4.88 ± 0.15
	120	3.89 ± 0.00	4.50 ± 0.25	5.50 ± 0.09
	180	4.76 ± 0.06	5.11 ± 0.23	6.44 ± 0.04
	240	5.18 ± 0.02	5.70 ± 0.01	7.05 ± 0.08

positive impact on the isolation of both investigated cannabinoids at the pressure of 100 and 300 bar, while this was not the case at the medium investigated pressure of 200 bar.

According to the obtained results, if the process is evaluate from the aspect of process efficiency and not from the aspect of extract quality, it can be concluded that the optimal conditions for the SC-CO_2 of cannabinoids from the areal hemp parts cv. Helena (the highest process efficiency) are pressure of 300 bar, temperature of 40 °C and extraction time of 120 min.

Omar et al. (2013) reported that the content of CBD and THC in SC-CO_2 extract from 13 different samples of *C. indica* obtained under different extraction conditions (pressure from 100 to 250 bar, temperature from 35 to 55 °C, co-solvent: ethanol in concentration from 0 to 40 %) were from 1 to 13 mg/g and from 4.5–324 mg/g, respectively. The CBD content in the extracts obtained from the industrial hemp cv. Helena is about 2.5 times higher than the CBD content obtained in the extracts reported by Kitryte et al. (2018) and about 100 times higher than the CBD content in the extract from *C. indica* reported by Omar et al. (2013). These comparisons indicate the high quality of Helena cultivar, but also the advantage regarding CBD in comparison to *C. indica*.

Comparing the extraction methods, it can be concluded that SC-CO_2 has proven to be the best method for extracting cannabinoids in their neutral and pharmacologically active form. Namely, the SC-CO_2 was more efficient than soxhlet extraction as the content of CBD in SC-CO_2 extract was about 2.5 times higher than in extract produced by soxhlet. Likewise, in comparison to the essential oil, extracts obtained by SC-CO_2 were characterized with the higher diversity of cannabinoids. Besides, the content of cannabidiol and cannabichromene were higher in the case of SC-CO_2 extracts. SC-CO_2 was also more powerful than microwave assisted extraction. Namely, according to our previously published study, the content of CBD was measured as 1.0420 mg/ml in the extract obtained under optimized process conditions of the same plant material (Drinić et al., 2019). But, in should be noticed that SC-CO_2 extraction also resulted in the detectable content of THC in the produced extracts (Fig. 4).

3.3.3. Conventional extraction (CE) of phenolic compounds from the material left after SC-CO_2 extraction

As supercritical extarction by carbon dioxide isolated only low polar constituents from the investigated material, the hydrophilic compounds remain in the plant material; therefore, further processing with aim of their isolation is meaningful to be set. To achieve this the plant material left after SC-CO_2 extraction was used for the extraction of hydrophilic compounds by CE method - modified maceration using 50 % ethanol as extraction solvent. This way set-up of the extraction process (SC-CO_2 extarction followed by CE) provides maximal utilization of the plant material, and minimal generation of the by-products and wastes. The EY, TP and TF of the liquid hemp extracts produced are present in the Table 6.

The extraction yield achieved in the CE of material left after SFE was

Table 4
Chemical composition of hemp total extract obtained by SC-CO_2 extraction.

	Compound	RI[a]	100 bar			200 bar			300 bar		
			40 °C	50 °C	60 °C	40 °C	50 °C	60 °C	40 °C	50 °C	60 °C
1.	*α*-thujene	925.8	/	/	/	/	/	/	/	/	0.26
2.	*β*-pinene	975	/	/	/	/	/	/	/	/	0.29
3.	*trans*-caryophyllene	1375.9	8.78	11.51	6.87	4.62	4.90	5.69	6.87	5.74	5.32
4.	*trans*-*α*-bergamotene	1383.2	0.83	1.35	0.97	0.56	0.58	0.69	0.80	0.68	0.60
5.	*cis*-*β*-farnesene	1395.2	0.98	1.69	1.15	0.58	0.68	0.79	0.88	0.83	0.72
6.	*α*-humulene	1406	2.47	3.29	1.98	1.33	1.42	1.63	2.21	1.75	1.58
7.	allo-aromadendrene	1413	/	0.31	/	/	/	/	/	/	/
8.	*β*-selinene	1436	0.61	0.99	0.67	/	0.40	0.39	0.55	0.50	0.39
9.	*α*-selinene	1443.1	0.38	0.61	0.43	/	/	/	0.36	0.22	0.26
10.	caryophyllene oxide	1523.6	1.14	4.18	4.23	0.81	1.00	0.87	1.14	1.28	0.67
11.	palmitic acid	1832.2	1.39	1.49	2.78	1.44	1.59	1.92	1.30	1.94	4.40
12.	phytol	1967.6	0.60	0.72	0.68	/	0.42	0.64	0.44	0.43	0.36
13.	*p*-pentyl acetophenone	2070.4	1.13	0.92	1.14	1.20	1.16	1.41	1.16	1.10	1.25
14.	cannabichromene	2126.2	0.33	0.32	/	/	/	/	/	/	/
15.	cannabidiol	2248.5	67.81	62.44	70.47	78.26	76.64	73.71	74.40	75.01	73.09
16.	tetrahydrocannabinol	2326.3	1.44	1.40	1.88	1.89	1.89	1.86	1.81	1.83	1.95
17.	cannabigerol	2368.3	0.47	0.40	0.56	0.60	0.62	0.58	0.58	0.58	0.59
18.	tetracosane	2457.9	0.62	0.50	/	0.52	0.49	0.54	0.45	0.46	0.44
19.	squalane	2587.2	1.54	1.20	0.88	1.08	1.11	1.44	1.06	1.10	1.29
20.	pentacosane	2648.8	6.91	4.86	3.37	5.51	5.44	6.05	4.63	5.05	4.96
21.	nonacosane	2891.9	1.96	1.23	1.16	1.58	1.67	1.79	1.36	1.49	1.58
22.	*α*-tocopherol	2965.6	0.61	0.60	0.79	/	/	/	/	/	/
Monoterpene hydrocarbons			/	/	/	/	/	/	/	/	0.55
Sesquiterpene hydrocarbons			15.19	23.92	16.29	7.91	8.97	10.07	12.81	11.01	9.53
Diterpenes			0.60	0.72	0.68	0.00	0.42	0.64	0.44	0.43	0.36
Triterpenes			1.54	1.20	0.88	1.08	1.11	1.44	1.06	1.10	1.29
Cannabinoids			71.17	65.48	74.05	81.95	80.32	77.56	77.96	78.51	76.88
Fatty acids and their esters			1.39	1.49	2.78	1.44	1.59	1.92	1.30	1.94	4.40
Hydrocarbons			9.49	6.59	4.52	7.61	7.59	8.38	6.44	7.00	6.99
Vitamins			0.61	0.60	0.79	/	/	/	/	/	/

[a] RI, retention indices as determined on HP-5 column using homologous series of C8-C30 alkanes.

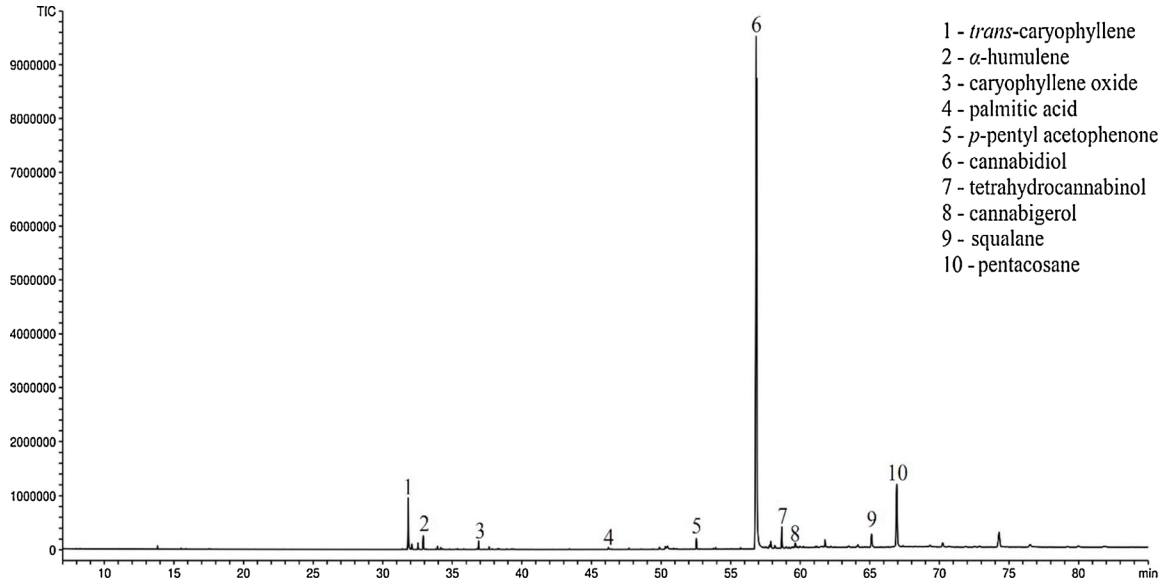

Fig. 3. GC/MS chromatogram of extract obtained from areal hemp part (assortment Helena) by application of SC-CO_2 extraction at 200 bar and 40 °C.

between 14.63–17.53 %. The highest yield was determined in the case of extract obtained from the plant material left after SFE at the pressure of 200 bar and the temperature of 50 °C, but there was no statistically significant difference when SFE temperature applied was 40 or 60 °C at the same pressure, when SFE temperature was 40 or 50 °C at the pressure of 300 bar and when SFE parameters was 100 bar and 40 °C.

TP content in the produced hemp extracts ranged from the 1.22–1.61 mg GAE/mL. The highest TP content was determined in the extract obtained from the plant material left after SFE at 100 bar and 40 °C, but there was not statistical difference when for extraction plant material left after SFE at 100 bar and 60 °C, 300 bar and 40 °C, and 300 bar and 60 °C was used. The lowest TP content was obtained when for the CE the plant material left after SFE at 100 bar and 50 °C was used, but there was no statistically significant difference between TP content in extracts obtained from plant material left after SFE at 200 bar at all applied temperatures or at 300 bar and 50 °C.

TF content in the produced hemp extracts was between 0.34 to 0.47 mg CE/mL. The highest was obtained in extract produced from the plant

Table 5
Cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC) content in total and fraction hemp extract obtained by SC-CO_2.

Extraction conditions		CBD (mg/g TE)[1]	CBD (mg/g dw)	THC (mg/g TE)	THC (mg/g dw)
100 bar, 40 °C	TE	163.11 ± 8.16 a	2.2411 ± 0.1121 h	6.58 ± 0.33 a	0.0904 ± 0.0045 i
	1h	99.74 ± 4.99 c	1.3357 ± 0.0668 k	3.77 ± 0.19 fgh	0.0505 ± 0.0025 l
	2h	78.03 ± 3.90 c-g	1.0450 ± 0.0523 lm	3.17 ± 0.16 ghi	0.0425 ± 0.0021 lm
	3h	34.23 ± 1.71 jkl	0.4585 ± 0.0229 o-r	1.56 ± 0.08 klm	0.0208 ± 0.0010 opq
	4h	24.89 ± 1.24 lm	0.3333 ± 0.0167 p-t	1.21 ± 0.06 k-o	0.0162 ± 0.0008 opq
100 bar, 50 °C	TE	98.09 ± 4.90 cd	0.5469 ± 0.0273 op	4.97 ± 0.25 cde	0.0277 ± 0.0014 no
	1h	50.24 ± 2.51 hij	0.1829 ± 0.0091 s-x	2.43 ± 0.12 ij	0.0088 ± 0.0004 qr
	2h	65.55 ± 3.28 f-i	0.2386 ± 0.0119 r-w	2.76 ± 0.14 i	0.0101 ± 0.0005 pqr
	3h	14.96 ± 0.75 lm	0.0545 ± 0.0027 vwx	0.95 ± 0.05 k-r	0.0035 ± 0.0002 r
	4h	11.35 ± 0.57 lm	0.0413 ± 0.0021 wx	0.81 ± 0.04 l-r	0.0029 ± 0.0001 r
100 bar, 60 °C	TE	71.84 ± 3.59 e-h	0.1782 ± 0.0089 s-x	4.17 ± 0.21 ef	0.0104 ± 0.0005 pqr
	1h	56.56 ± 2.83 g-j	0.0942 ± 0.0047 u-x	1.80 ± 0.09 jk	0.0022 ± 0.0001 r
	2h	34.03 ± 1.70 jkl	0.0567 ± 0.0028 vwx	1.04 ± 0.05 k-q	0.0013 ± 0.0001 r
	3h	3.21 ± 0.16 m	0.0054 ± 0.0003 x	0.11 ± 0.01 r	0.0001 ± 0.0000 r
	4h				
200 bar, 40 °C	TE	149.22 ± 7.46 a	6.4678 ± 0.3234 b	5.96 ± 0.30 ab	0.2585 ± 0.0129 d
	1h	84.60 ± 4.23 c-f	3.6921 ± 0.1846 e	3.96 ± 0.20 fg	0.1729 ± 0.0086 g
	2h	35.25 ± 1.76 jkl	1.5383 ± 0.0769 jk	1.52 ± 0.08 klm	0.0665 ± 0.0033 jk
	3h	20.17 ± 1.01 lm	0.8802 ± 0.0440 lm	0.77 ± 0.04 m-r	0.0334 ± 0.0017 mn
	4h	9.09 ± 0.45 m	0.3969 ± 0.0198 p-s	0.41 ± 0.02 o-r	0.0177 ± 0.0009 opq
200 bar, 50 °C	TE	78.33 ± 3.92 c-g	3.3525 ± 0.1676 f	5.49 ± 0.27 bc	0.2351 ± 0.0118 e
	1h	47.83 ± 2.39 ijk	2.1654 ± 0.1083 h	3.66 ± 0.18 fgh	0.1656 ± 0.0083 g
	2h	23.89 ± 1.19 lm	1.0816 ± 0.0541 l	1.70 ± 0.09 jk	0.0771 ± 0.0039 j
	3h	14.79 ± 0.74 lm	0.6696 ± 0.0335 no	0.95 ± 0.05 k-r	0.0428 ± 0.0021 lm
	4h	6.59 ± 0.33 m	0.2984 ± 0.0149 q-u	0.37 ± 0.02 o-r	0.0169 ± 0.0008 opq
200 bar, 60 °C	TE	94.19 ± 4.71 cde	4.4074 ± 0.2204 d	5.60 ± 0.28 bc	0.2622 ± 0.0131 d
	1h	48.72 ± 2.44 h-k	1.9161 ± 0.0958 i	3.75 ± 0.19 fgh	0.1474 ± 0.0074 h
	2h	27.30 ± 1.37 klm	1.0738 ± 0.0537 l	1.68 ± 0.08 jkl	0.0662 ± 0.0033 jk
	3h	12.11 ± 0.61 lm	0.4763 ± 0.0238 opq	0.68 ± 0.03 m-r	0.0266 ± 0.0013 no
	4h	11.55 ± 0.58 lm	0.4542 ± 0.0227 o-r	0.55 ± 0.03 n-r	0.0215 ± 0.0011 op
300 bar, 40 °C	TE	120.37 ± 54.44 b	7.7582 ± 0.3879 a	5.09 ± 1.99 cd	0.3170 ± 0.0159 b
	1h	61.11 ± 3.06 ghi	3.7622 ± 0.1881 e	2.93 ± 0.15 hi	0.0107 ± 0.0005 pqr
	2h	71.33 ± 3.57 e-h	1.7190 ± 0.0860 j	3.08 ± 0.15 hi	0.0112 ± 0.0006 pqr
	3h	12.55 ± 0.63 lm	0.8494 ± 0.0425 mn	0.46 ± 0.02 n-r	0.0017 ± 0.0001 r
	4h	7.25 ± 0.36 m	0.2609 ± 0.0130 q-w	0.30 ± 0.02 pqr	0.0011 ± 0.0001 r
300 bar, 50 °C	TE	86.98 ± 4.35 c-f	4.4917 ± 0.2246 cd	5.65 ± 0.28 bc	0.2920 ± 0.0146 c
	1h	56.83 ± 2.84 g-j	2.7505 ± 0.1375 g	4.32 ± 0.22 def	0.2093 ± 0.0105 f
	2h	21.20 ± 1.06 lm	1.0263 ± 0.0513 lm	1.32 ± 0.07 k-n	0.0637 ± 0.0032 k
	3h	6.50 ± 0.32 m	0.3144 ± 0.0157 q-u	0.37 ± 0.02 o-r	0.0177 ± 0.0009 opq
	4h	3.12 ± 0.16 m	0.1510 ± 0.0076 t-x	0.21 ± 0.01 qr	0.0101 ± 0.0005 pqr
300 bar, 60 °C	TE	76.12 ± 3.81 d-g	4.6756 ± 0.2338 c	5.69 ± 0.28 bc	0.3493 ± 0.0175 a
	1h	65.54 ± 3.28 f-i	3.8341 ± 0.1917 e	4.41 ± 0.22 def	0.2581 ± 0.0129 d
	2h	24.81 ± 1.24 lm	1.4512 ± 0.0726 k	1.20 ± 0.06 k-p	0.0702 ± 0.0035 jk
	3h	7.45 ± 0.37 m	0.4356 ± 0.0218 pqr	0.37 ± 0.02 o-r	0.0219 ± 0.0011 op
	4h	4.68 ± 0.23 m	0.2738 ± 0.0137 q-v	0.21 ± 0.01 qr	0.0121 ± 0.0006 pqr

[1] Means followed by different letters are significantly different according to the post hoc Duncan's test at level $P < 0.05$.

material left after SFE at 100 bar and 60 °C, but there was no statistically significant difference with extract obtained from the plant material left after SFE at 300 bar and 40 °C. The lowest TF content was measured in the extract obtained from the plant material left after SC-CO_2 extraction at 300 bar and 50 and 60 °C.

Appropriate choice of SC-CO_2 extraction parameters with respect to TP and TF content in CE extract of plant material after SFE would be 100 bar and 60 °C. Optimal SC-CO_2 extraction parameters for obtained the SC-CO_2 extraction extract with respect to extraction yield and cannabinoids content were 300 bar and 40 °C. TP and TF content in CE extract of plant material after SC-CO_2 extraction at 300 bar and 40 °C were statistical insignificant different from TP and TF content obtained in CE extract of plant material after SC-CO_2 extraction at 100 bar and 60 °C. Consequently, the best choice of SC-CO_2 extraction parameters for both extractions, SC-CO_2 extraction and CE, would be 300 bar and 40 °C.

According to our previous study, in the hemp extracts (cultivar Helena, same plant material as it is used in this study) obtained by CE at the room temperature for 24 h with 50 % ethanol as the extraction solvent, applying solid/liquid ratio of 1:20, achieved content of TP and TF was 0.46 mg GAE/mL and 0.26 mg CE/mL, respectively (Drinić et al., 2018). According to previously mentioned, it can be concluded that SC-CO_2 extraction have positive influence on the extraction of TP and TF from the hemp. Thus, the combination of these two extraction techniques, SC-CO_2 extraction and CE, enables maximum utilization of the hemp, whereby two high-value types of extracts are obtained, first containing lipophilic low-polar constituents and second one containing polar constituents such are phenolics.

André et al. (2020) investigate the influence of different sowing densities as well as growth plant phase on the chemical composition of the inflorescences of eight different hemp cultivars: the six monoecious cultivars Felina 32, Futura 75, Fedora 17, Fibror 79, Santhica 27 and Santhica 70, as well as the two dioicous cultivars KC Virtus and Finola. TP content for full flowering plant were 0.84 mg GAE/mL for Fedora 17, 1.17 mg GAE/mL for Felina 32, from 0.58 to 1.07 mg GAE/mL for Fibror 79, 1.24 mg GAE/mL for Finola, 0.83 mg GAE/mL for Futura 75, from 0.38 to 1.26 mg GAE/mL for KC Virtus, 1.07 mg GAE/mL for Santhica 27, and 0.72 mg GAE/mL for Santhica 70. Comparing the TP content in the hemp extracts obtained in the study by André et al. (2020) with the TP content of hemp cv. Helena extracts obtained in this study, it can be concluded that cultivar Helena present a valuable genetic material considering phenolic constituents to.

3.4. SWE

Up to now SWE has been used for the extraction of many plant materials: *Rosmarinus officinalis* (Ibanez et al., 2003), *Satureja hortensis*

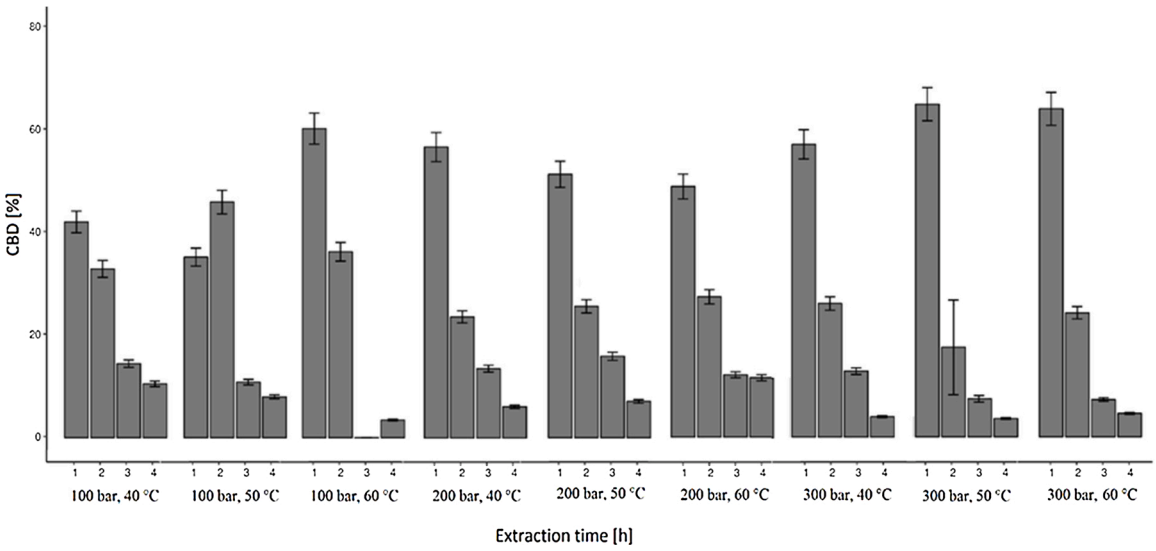

Fig. 4. Percent of isolated cannabidiol (CBD) after each hour of extraction.

Table 6
The extraction yield (EY), content of total phenols (TP), and total flavonoids (TF) in extract obtained by CE of plant material after SC-CO_2 extraction (SFE).

SFE parameters		EY [%][1]	TP [mg GAE/mL]	TF [mg CE/mL]
100 bar	40 °C	17.44 ± 0.87a	1.61 ± 0.08a	0.3900 ± 0.02 cd
	50 °C	15.52 ± 0.46bc	1.22 ± 0.06e	0.4000 ± 0.02bc
	60 °C	14.63 ± 0.43c	1.54 ± 0.08ab	0.4700 ± 0.02a
200 bar	40 °C	16.54 ± 0.48ab	1.39 ± 0.07b-e	0.3600 ± 0.02 cd
	50 °C	17.53 ± 0.51a	1.32 ± 0.07cde	0.3900 ± 0.02 cd
	60 °C	16.75 ± 0.49ab	1.35 ± 0.07b-e	0.3800 ± 0.02 cd
300 bar	40 °C	16.07 ± 0.18abc	1.51 ± 0.03abc	0.4500 ± 0.02ab
	50 °C	16.79 ± 0.49ab	1.27 ± 0.06de	0.3400 ± 0.02d
	60 °C	15.82 ± 0.46bc	1.45 ± 0.07a-d	0.3400 ± 0.02d

[1] Means followed by different letters are significantly different according to the post hoc Duncan's test at level $P < 0.05$.

Table 7
Influence of temperature in SWE on the extraction yield (EY), content of total phenols (TP), total flavonoids (TF), and cannabidiol (CBD).

T [°C]	EY [%][1]	TP [mg GAE/mL]	TF [mg CE/mL]	CBD [mg/mL]
120	21.59 ± 0.11 e	1.00 ± 0.03 f	0.48 ± 0.01 e	0.0145 ± 0.0014 ab
140	24.44 ± 0.28 d	1.28 ± 0.03 e	0.58 ± 0.01 d	0.0183 ± 0.0025 a
160	27.02 ± 0.62 c	1.58 ± 0.01 d	0.67 ± 0.01 c	0.0156 ± 0.0074 a
180	29.71 ± 0.29 b	1.73 ± 0.06 c	0.72 ± 0.01 b	0.0138 ± 0.0017 ab
200	32.41 ± 1.23 a	2.10 ± 0.10 b	0.79 ± 0.01 a	0.0063 ± 0.0015 bc
220	29.49 ± 0.19 b	2.26 ± 0.04 a	0.81 ± 0.01 a	0.0039 ± 0.0002 c

[1] Means followed by different letters are significantly different according to the post hoc Duncan's test al level $P < 0.05$.

(Vladić et al., 2017), *Marrubium vulgare* (Gavarić et al., 2019), *Syzygium aromaticum* (Clifford et al., 1999), *Origanum vulgare* (Soto Ayala and Luque de Castro, 2001), *Foeniculum vulgare* (Gamiz-Garcıa and Luque de Castro, 2000). The physical and chemical properties of water can be manipulated by changing the SWE parameters (pressure and temperature). Water in subcritical states remains in liquid states with the possibility to extract the compounds of different polarity: from polar (by applying lower temperatures) to nonpolar (by applying higher temperatures) (Ramos et al., 2002). However, the use of high temperature is usually followed by degradation and transformation of some compounds leading to the formation of various beneficial or harmful compounds. According to literature data up to now SWE of hemp areal parts was not investigated, therefore it was applied in the study for preparation of hemp extracts. In the investigation the temperature as dominant parameter in SWE was set as variable process parameter, while time and pressure were constant. Totally 6 different extracts were prepared in the range of process temperatures from 120 to 200 °C. The extraction yield, TP and TF, as well as the content of CBD and THC were examined in the obtained extracts and results were present in Table 7.

Extraction yield obtained by application of SWE was in the ranged from 21.59 to 32.41 %. Statistical difference in extraction yield was observed in all extracts, except for extracts obtained at 180 and 220 °C for which no statistically significant difference in the yield was found. The lowest yield was obtained in the extract where lowest extraction temperature was applied. The extraction yield was increase with the increasing extraction temperature up to 200 °C, while with further increasing to 220 °C yield was decreased. The decrease in the yield at a temperature of 220 °C is probable due to degradation of extracted compound. Several authors reported on the degradation of certain compounds in SWE at the temperature of 220 °C and higher (Khuwijitjaru et al., 2004; Lamoolphak et al., 2006; Sereewatthanawut et al., 2007). Comparing the EY obtained in SWE with other extraction techniques used in this study it can be concluded that SWE gained the highest EYs. Having in mind the fact that other applied extraction techniques are suitable for the extraction the low polar (Soxhlet and SFE) or polar (CE) compounds, the highest yield in the case of SWE can be attributed to the technique possibility to isolate both low polar and polar compounds.

The content of TP and TF in SWE hemp extracts was in the range from 1.00–2.26 mg GAE/mL and from 0.48 to 0.81 mg CE/mL, respectively. The TP and TF content increased with the increasing temperature and the highest value was reached at the highest applied extraction temperature of 220 °C. TP content was statistically different in all obtained extract, while TF content was significantly different in the extracts obtained at the temperature from 120 to 180 °C, while between extracts obtained at 200 and 220 °C there was no statistical difference. Increase in the phenol content with the increase of the temperature in SWE was observed in many studies. He et al. (2012) examined the influence of temperature in the range from 80 to 280 °C on the subcritical extraction of phenols from pomegranate. According to the results of this study the TP content was increased with the increasing temperature from 80 to

220 °C, while further temperature increase lead to the decrease in the TP content. It can be assumed that increasing TP and TF content with the increased temperature is caused due to the formation of the new phenolic constituents. Namely, previous studies reported that new phenolic compounds, such as phenolic acids, can be formed by degradation of lignin, cellulose and hemicellulose under conditions of high temperature and pressure (Wiboonsirikul et al., 2007, 2008). An increase in the phenol content due to the lignin degradation was also observed in the study of Pourali et al. (2010), who examined the effect of temperature on the SWE of rice bran. According to the study by Pourali et al. (2010) the TP content in SWE extracts of rice bran increase with increasing the temperature from 150 to 220 °C after which it remained constant with the further increasing temperature. Some of the phenolic acids that are formed as a result of the lignin degradation are caffeic, ferulic, gallic, sinapinic, *p*-coumaric, p-hydroxybenzoic, syringic, vanillic and others. The raw hemp fiber contains about 67–78 % cellulose, 16–19 % hemicellulose, and 2.9–3.3 % lignin (Buschle Diller et al., 1999). The phenolic acids and aldehydes identified in hemp lignin were vanillin, syringaldehyde, p-hydroxybenzaldehyde, vanillic acid, syringic acid, p-coumaric acid, acetosyringone, and gallic acid (Gandolfi et al., 2013). It can be assumed that in some point of the temperature increase in SWE this content of lignin is the reason of the TP increase.

The TP and TF content obtained in SWE extracts was about 1.5 times higher than in extracts obtained by CE after SC-CO_2 extraction, this leads to the conclusion that the SWE is more appropriate extraction techniques for preparation of hemp extracts with higher content of phenolic compounds. But application of this process for phenolic constituents' isolation instead of CE after SC-CO_2 extraction requiring higher investments costs as well as more extreme process conditions and therefore more demanding maintenance.

According to the obtained results, the THC values for all obtained extracts were under the detection limit, therefore it can be concluded that this extraction technology or extreme process temperature conditions are not suitable for the isolation of this kind of compound. The CBD content in the obtained SWE hemp extracts was in the range from 0.0039 to 0.0183 mg/mL. The highest CBD content was obtained at the temperature of 140 °C and did not differ statistically significant from the content obtained in the extract at temperature of 120, 160, and 180 °C. Influence of extraction time (5, 10, 15, 30, and 40 min) at constant temperature of 140 °C was examined too, but results per time did not differ statistically significant. CBD content in this way obtained extracts was in range from 0.0138 to 0.0210 mg/mL. Cannabinoids decarboxylation occurs at the high temperature and because of that higher cannabinoid content were expected in SWE extracts. Wianowska et al. (2015) studied the influence of temperature and time in pressure liquid extraction (PLE) on extraction of THCA and its decarboxylated form, THC, as well as further oxidation of THC in CBN. Although temperature in PLE was high (150 °C), decarboxylation of THCA to THC was low. The low CBD content, as well as THC, in the studied SWE extracts obtained from areal parts of the hemp cv. Helena, is probably due to the low decarboxylation of cannabinoid acids into their corresponding decarboxylated forms under the conditions of elevated pressure and temperature, as noted in the study by Wianowska et al. (2015). The CBD content in the SWE extract was significantly lower than in the extracts obtained by the others extraction techniques. Namely, according to the obtained and previous published results (Drinić et al., 2019), 64.40 mg/g CBD can be achieved by soxhlet extraction, from 71.84–163.11 mg/g by SC-CO_2 extraction, and from 0.22 to 1.84 mg/ml by microwave-assisted extraction. How the CBD is low polar compound, these results were expected. Thus, SWE is not appropriate technique to be selected for this kind of hemp bioactive compound isolation.

4. Conclusion

Results of the study showed that the Helena commercial variety can be designate as a variety with the great potential for future utilization due to its chemical composition. Compared to the other variety it is characterized with the higher content of nonpsychoactive CBD and the lower content of psychoactive THC. Comparing the content of essential oil with the different hemp variety, Helena showed higher or similarly content of essential oil with the high concentration of sesquiterpenes, which may play a highly significant role in the human health. Hemp cv. Helena essential oil contained higher CBD content comparing to the other hemp variety. Different extraction techniques applied for the isolation of bioactives from the investigated hemp cv. Helena resulted in the preparation of extracts of different quality, in mean number of bioactive constituents and their content. Therefore, depending on the desired characteristics of the hemp extracts, different extraction technique should be applied. Soxhlet extraction showed good properties for the isolation of cannabinoids, but it is not technique of the choice for the isolation of hemp sesquiterpene bioactives. SWE proved to be effective for the extraction of hemp polyphenols, while the CBD content in this way obtained extracts was low. Results have showed that the most powerful process for hemp cv. Helena bioactive compounds isolation was SC-CO_2 extraction followed by CE. These two coupled extraction techniques showed adequate results regarding extraction of low polar bioactives, such as cannabinoids and terpenes compounds, as well as polar compounds, such as polyphenols. SC-CO_2 extraction of hemp resulted in the production of extract of the highest CBD content. The same extract contained a variety of other valuable bioactive hemp sesquiterpenes. Moreover, extraction of hemp left after SC-CO_2 extraction resulted in the production of extracts rich in hemp phenolics bioactives. Both techniques used in coupled process are already aintensively applied on the industrial level, what gives this way set process the highest potential to be commercially applied for hemp cv. Helena utilization.

CRediT authorship contribution statement

Zorica Drinić: Investigation, Writing - original draft. **Jelena Vladic:** Investigation. **Anamarija Koren:** Resources, Writing - review & editing. **Tijana Zeremski:** Investigation. **Nadežda Stojanov:** Investigation. **Milan Tomić:** Investigation. **Senka Vidović:** Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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References

Adams, R.P., 2007. Identification of Essential Oil Components byGas Chromatography/ Mass Spectrometry. Allured Publishing Corporation. Google-Books-ID: 9ut3PQAACAAJ.

Agarwal, C., Mathe, K., Hofmann, T., Csoka, L., 2018. Ultrasound-assisted extraction of cannabinoids from *Cannabis sativa* L. optimized by response surface methodology. J. Food Sci. 83 (3), 700–711. https://doi.org/10.1111/1750-3841.14075.

Aladić, K., Jarni, K., Barbir, T., Vidović, S., Vladić, J., Bilić, M., Jokić, S., 2015. Cold pressing and supercritical CO2 extraction of hemp (*Cannabis sativa*) seed oil. Ind. Crops Prod. 76, 472–478. https://doi.org/10.15255/CABEQ.2013.1895.

Andre, C.M., Larondelle, Y., Evers, D., 2010. Dietary antioxidants and oxidative stress from a human and plant perspective: a review. Curr. Nutr. Food Sci. 6, 2–12. https://doi.org/10.2174/157340110790909563.

Andre, C.M., Hausman, J.-F., 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.

André, A., Leupin, M., Kneubühl, M., Pedan, V., Chetschik, I., 2020. Evolution of the polyphenol and terpene content, antioxidant activity and plant morphology of eight diferent fiber-type cultivars of Cannabis sativa L. cultivated at three sowing densities. Plants 9 (12), 1740. https://doi.org/10.3390/plants9121740.

Atalay, S., Jarocka-Karpowicz, I., Skrzydlewska, E., 2020. Antioxidative and anti-inflammatory properties of Cannabidiol. Antioxidants 9 (1), 21. https://doi.org/10.3390/antiox9010021.

Attard, T.M., Bainier, C., Reinaud, M., Lanot, A., McQueen-Mason, S.J., Hunt, A.J., 2018. Utilisation of supercritical fluids for the effective extraction of waxes and cannabidiol (CBD) from hemp wastes. Ind. Crops Prod. 112, 38–46. https://doi.org/10.1016/j.indcrop.2017.10.045.

Baldino, L., Scognamiglio, M., Reverchon, E., 2020. Supercritical fluid technologies applied to the extraction of compounds of industrial interest from Cannabis sativa L. And to their pharmaceutical formulations: a review. J. Supercrit. Fluids 165, 104960. https://doi.org/10.1016/j.supflu.2020.104960.

Bócsa, I., Karus, M., 1998. The Cultivation of Hemp: Botany, Varieties, Cultivation and Harvesting. Hemptech, Sebastopol, CA.

Brenneisen, R., 2007. Chemistry and analysis of phytocannabinoids and other Cannabis constituents. In: ElSohly, M.A. (Ed.), Forensic Science and Medicine: Marijuana and the Cannabinoids. Humana Press Inc, Totowa, New Jersey. https://doi.org/10.1007/978-1-59259-947-9_2.

Brighenti, V., Pellati, F., Steinbach, M., Maran, D., Benvenuti, S., 2017. Development of a new extraction technique and HPLC method for the analysis of non-psychoactive cannabinoids in fibretype *Cannabis sativa* L.(hemp). J. Pharm. Biomed. Anal. https://doi.org/10.1016/j.jpba.2017.05.049.

Buschle Diller, G., Fanter, C., Loth, F., 1999. Structural changes in hemp fibers as a result of enzyme systems. Text. Res. J. 69, 244–251. https://doi.org/10.1177/004051759906900403.

Callaway, J.C., 2008. A more reliable evaluation of hemp THC levels is necessary and possible. J. Ind. Hemp 13, 117–144. https://doi.org/10.1080/15377880802391142.

Clifford, A.A., Basile, A., Al-Saidi, S.H., 1999. A comparison of the extraction of clove buds with supercritical carbon dioxide and superheated water. Fresenius J. Anal. Chem. 364, 635–637. https://doi.org/10.1007/s002160051400.

Da Porto, C., Voinovich, D., Decorti, D., Natolinoa, A., 2012. Response surface optimization of hemp seed (*Cannabis sativa* L.) oil yield and oxidation stability by supercritical carbon dioxide extraction. J. Supercrit. Fluids 68, 45–51. https://doi.org/10.1016/j.supflu.2012.04.008.

Da Porto, C., Decorti, D., Natolino, A., 2014. Separation of aroma compounds from industrial hemp inflorescences (*Cannabis sativa* L.) by supercritical CO2 extraction and on-line fractionation. Ind. Crops Prod. 58, 99–103. https://doi.org/10.1016/j.indcrop.2014.03.042.

Devi, V., Khanam, S., 2019. Study of ω-6 linoleic and ω-3 α-linolenic acids of hemp (*Cannabis sativa*) seed oil extracted by supercritical CO2 extraction: CCD optimization. J. Environ. Chem. Eng. 7, 102818 https://doi.org/10.1016/j.jece.2018.102818.

Devinsky, O., Marsh, E., Friedman, D., 2016. Cannabidiol in patients with treatment-resistant epilepsy - Authors' reply. Lancet Neurol. 15 (6), 545–546. https://doi.org/10.1016/S1474-4422(16)00120-4.

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

Drinić, Z., Vladić, J., Koren, A., Zeremski, T., Stojanov, N., Kiprovski, B., Vidović, S., 2019. Microwave-assisted extraction of cannabinoids and antioxidants from Cannabis sativa aerial parts and process modeling. Chemical Technology and Biotechnology 95 (3), 831–839. https://doi.org/10.1002/jctb.6273.

Fathordoobady, F., Singh, A., Kitts, D.D., Pratap Singh, A., 2019. Hemp (Cannabis sativa L.) extract: anti-microbial properties, methods of extraction, and potential oral delivery. Food Rev. Int. 35, 664–684.

Frassinetti, S., Moccia, E., Caltavuturo, L., Gabriele, M., Longo, V., Bellani, L., Giorgi, G., Giorgetti, L., 2018. Nutraceutical potential of hemp (Cannabis sativa L.) seeds and sprouts. Food Chem. 262, 56–66.

Gallo-Molina, A.C., Castro-Vargas, H.I., Garzón-Méndez, W.F., Martínez Ramírez, J.A., Rivera Monroy, J.Z., King, J.W., Parada-Alfonso, F., 2019. Extraction, isolation and purification of tetrahydrocannabinol from the *Cannabis sativa* L. Plant using supercritical fluid extraction and solid phase extraction. J. Supercrit. Fluids. https://doi.org/10.1016/j.supflu.2019.01.020.

Gamiz-Garcıa, L., Luque de Castro, M.D., 2000. Continuous subcritical water extraction of medicinal plant essential oil: comparison with conventional techniques. Talanta 51, 1179–1185. https://doi.org/10.1016/S0039-9140(00)00294-0.

Gandolfi, S., Ottolina, G., Riva, S., Fantoni, G.P., Patel, I., 2013. Complete chemical analysis of carmagnola hemp hurds and structural features of its components. Bioresources 8 (2), 2641–2656. https://doi.org/10.15376/biores.8.2.2641-2656.

Gavarić, A., Vladić, J., Ambrus, R., Jokić, S., Szabó-Révész, P., Tomić, M., Blažić, M., Vidović, S., 2019. Spray drying of a subcritical extract using Marrubium vulgare as a method of choice for obtaining high quality powder. Pharmaceutics 11 (10), 523. https://doi.org/10.3390/pharmaceutics11100523.

Hampson, A.J., Grimaldi, M., Axelrod, J., Wink, D., 1998. Cannabidiol and Δ^9-tetrahydrocannabinol are neuroprotective antioxidants. Proc. Natl. Acad. Sci. U.S.A. 95, 8268–8273. https://doi.org/10.1073/pnas.95.14.8268.

Harborne, J.B., 1999. Classes and functions of secondary productsfrom plants. In: Walton, N.J., Brown, D.E. (Eds.), Chemicalsfrom Plants: Perspectives on Plant Secondary Products. WorldScientific, pp. 1–25.

He, L., Zhang, X., Xu, H., Xu, C., Yuan, F., Knez, Ž., Novak, Z., Gao, Y., 2012. Subcritical water extraction of phenolic compounds from pomegranate (Punica granatum L.) seed residues and investigation into their antioxidant activities with HPLC–ABTS•+ assay. Food Bioprod. Process. 90, 215–223. https://doi.org/10.1016/j.fbp.2011.03.003.

Hochmuth, D., Hamburg, Germany, 2006. Massfinder 3: Softwarefor GC/MS Interpretation and Presentation. Mass SpectralLibrary Administration.

Ibanez, E., Kuvatova, A., Senorans, F.J., Cavero, S., Reglero, G., Hawthorne, S.B., 2003. Subcritical water extraction of antioxidant compounds from rosemary plants. J. Agric. Food Chem. 51, 375–382. https://doi.org/10.1021/jf025878j.

Izzo, L., Castaldo, L., Narváez, A., Graziani, G., Gaspari, A., Rodríguez-Carrasco, Y., Ritieni, A., 2020. Analysis of phenolic compounds in commercial Cannabis sativa L. Inflorescences using UHPLC-Q-Orbitrap HRMS. Molecules 25 (3), 631. https://doi.org/10.3390/molecules25030631.

Ju, Y.H., Huynh, L.H., Kasim, N.S., Guo, T.J., Wang, J.H., Fazary, A.E., 2011. Analysis of soluble and insoluble fractions of alkali and subcritical water treated sugarcane bagasse. Carbohydr. Polym. 83, 591–599. https://doi.org/10.1016/j.carbpol.2010.08.022.

Juárez, M.J., Vladic, J., Bolado Rodríguez, S., Vidovic, S., 2020. Sequential valorisation of microalgae biomass grown in pig manure treatment photobioreactors. Algal Res. 50 https://doi.org/10.1016/j.algal.2020.101972.

Kähkönen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.-P., Pihlaja, K., Kujala, T.S., Heinonen, M., 1999. Antioxidant activity of plant extracts containing phenolic compounds. J. Agric. Food Chem. 47, 3954–3962. https://doi.org/10.1021/jf990146l.

Khuwijitjaru, P., Fujii, T., Adachi, S., Kimura, Y., Matsuno, R., 2004. Kinetics on the hydrolysis of fatty acid esters in subcritical water. Chem. Eng. J. 99 (1), 1–4. https://doi.org/10.1016/j.cej.2003.08.002.

Kitrytė, V., Bagdonaitė, D., Venskutonis, P.R., 2018. Biorefining of industrial hemp (Cannabis sativa L.) threshing residues into cannabinoid and antioxidant fractions by supercritical carbon dioxide, pressurized liquid and enzyme-assisted extractions. Food Chem. 267, 420–429. https://doi.org/10.1016/j.foodchem.2017.09.080.

Klingeren, B.V., Ham, M.T., 1976. Antibacterial activity of Δ9-tetrahydrocannabinol and cannabidiol. Antonie Van Leeuwenhoek 42, 9–12. https://doi.org/10.1007/BF00399444.

Lamoolphak, W., Goto, M., Sasaki, M., Suphantharika, M., Muangnapoh, C., Prommuag, C., Shotipruk, A., 2006. Hydrothermal decomposition of yeast cells for production of proteins and amino acids. J. Hazard. Mater. 137 (3), 1643–1648. https://doi.org/10.1016/j.jhazmat.2006.05.029.

Lewis-Bakker, M.M., Yang, Y., Vyawahare, R., Kotra, L.P., 2019. Extractions of medical cannabis cultivars and the role of decarboxylation in optimal receptor responses. Cannabis Cannabinoid Res. https://doi.org/10.1089/can.2018.0067.

McPartland, J.M., 1984. Pathogenicity of Phomopsis ganjae on Cannabis sativa and the fungistatic effect of cannabinoids produced by the host. Mycopathologia 87, 149–153. https://doi.org/10.1007/BF00436900.

Mediavilla, V., Steinemann, S., 1997. Essential oil of Cannabis sativa L. Strains. Journal of the international hemp association 4, 80–82.

Mkpenie, V., Essien, E., Udoh, I., 2012. Effect of extraction conditions on total polyphenol contents, antioxidant and antimicrobial activities of Cannabis sativa L. Electronic Journal of Environmental. Agricultural and Food Chemistry 11 (4), 300–307.

Naffati, A., Vladić, J., Pavlić, B., Radosavljević, R., Gavarić, A., Vidović, S., 2017. Recycling of filter tea industry by-products: application of subcritical water extraction for recovery of bioactive compounds from A. Uva-ursi herbal dust. J. Supercrit. Fluids 121, 1–9. https://doi.org/10.1016/j.supflu.2016.11.010.

Nagy, D.U., Cianfaglione, K., Maggi, F., Sut, S., Dall'Acqua, S., 2019. Chemical characterization of leaves, male and female flowers from spontaneous Cannabis (Cannabis sativa L.) growing in Hungary. Chem. Biodivers. 16, e1800562.

Naz, S., Hanif, M.A., Bhatti, H.N., Ansari, T.M., 2017. Impact of supercritical fluid extraction and traditional distillation on the isolation of aromatic compounds from Cannabis indica and *Cannabis sativa*. Journal of Essential Oil-Bearing Plants 20 (1), 175–184. https://doi.org/10.1080/0972060X.2017.1281766.

Omar, J., Olivares, M., Alzaga, M., Etxebarria, N., 2013. Optimisation and characterisation of marihuana extracts obtained by supercritical fluid extraction and focused ultrasound extraction and retention time locking GC-MS. J. Sep. Sci. 36, 1397–1401. https://doi.org/10.1002/jssc.201201103.

Perrotin-Brunel, H., Perez, P.C., van Roosmalen, M.J.E., van Spronsen, J., Witkamp, G.-J., Peters, C.J., 2010. Solubility of Δ^9-tetrahydrocannabinol in supercritical carbon dioxide: experiments and modeling. J. Supercrit. Fluids 52, 6–10. https://doi.org/10.1016/j.supflu.2009.12.001.

Poortman-van der Meer, A.J., Huizer, H., 1999. A contribution to the improvement of accuracy in the quantitation of THC. Forensic Sci. Int. 101, 1–8. https://doi.org/10.1016/s0379-0738(99)00004-3.

Pourali, O., Asghari, F.S., Yoshidaa, H., 2010. Production of phenolic compounds from rice bran biomass under subcritical water conditions. Chem. Eng. J. 160, 259–266. https://doi.org/10.1016/j.cej.2010.02.057.

Ramos, L., Kristenson, E.M., Brinkman, U.A.T., 2002. Current use of pressurised liquid extraction and sub-critical water extraction in environmental analysis. J. Chromatogr. A 975, 3–29. https://doi.org/10.1016/S0021-9673(02)01336-5.

Reverchon, E., De Marco, I., 2006. Supercritical fluid extraction and fractionation of natural matter. J. Supercrit. Fluids 38, 146–166. https://doi.org/10.1016/j.supflu.2006.03.020.

Ribeiro Grijó, D., Vieitez Osorio, I.A., Cardozo-Filho, L., 2019. Supercritical extraction strategies using CO2 and ethanol to obtain cannabinoid compounds from cannabis hybrid flowers. J. Co2 Util. 30, 241–248. https://doi.org/10.1016/j.jcou.2018.09.022.

Romano, L.L., Hazekamp, A., 2014. Cannabis Oil: chemical evaluation of an upcoming cannabis-based medicine. Cannabinoids 1 (1), 1–11.

Rovetto, L.J., Aieta, N.V., 2017. Supercritical carbon dioxide extraction of cannabinoids from *Cannabis sativa* L. J. Supercrit. Fluids 129, 16–27. https://doi.org/10.1016/j.supflu.2017.03.014.

Russo, E.B., 2011. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Ther. Clin. Risk Manag. 163 (7), 1344–1364. https://doi.org/10.1111/j.1476-5381.2011.01238.x.

Schier, Alexandre Rafaelde Mello, Ribeiro, Natalia Pinhode Oliveira, Silva, Adriana Cardosode Oliveira, Hallak, JaimeEduardo Cecílio, Crippa, JoséAlexandre S., Nardi, Antonio E., Zuardi, Antonio Waldo., 2012. Cannabidiol, a Cannabis sativa constituent, as an anxiolytic drug. Brazilian Journal of Psychiatry 34 (1), 104–110. https://doi.org/10.1590/S1516-44462012000500008.

Sereewatthanawut, I., Prapintip, S., Watchiraruji, K., Goto, M., Sasaki, M., Shotipruk, A., 2007. Extraction of protein and amino acids from deoiled rice bran by subcritical water hydrolysis. Bioresour. Technol. 99 (3), 555–561. https://doi.org/10.1016/j.biortech.2006.12.030.

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

Soto Ayala, R., Luque de Castro, M.D., 2001. Continuous subcritical water extraction as a useful tool for isolation of edible essential oils. Food Chem. 75, 109–113. https://doi.org/10.1016/S0308-8146(01)00212-6.

Vidović, S., Mujić, I., Zeković, Z., Lepojević, Ž., Milošević, S., Jokić, S., 2011. Extraction of fatty acids from boletus edulis by subcritical and supercritical carbon dioxide. J. Am. Oil Chem. Soc. 88, 1189–1196. https://doi.org/10.1007/s11746-011-1772-5.

Vladić, J., Canli, O., Pavlić, B., Zeković, Z., Vidović, S., Kaplan, M., 2017. Optimization of Satureja montana subcritical water extraction process and chemical characterization of volatile fraction of extracts. J. Supercrit. Fluids 120 (1), 86–94. https://doi.org/10.1016/j.supflu.2016.10.016.

Wianowska, D., Dawidowicz, A.L., Kowalczyk, M., 2015. Transformations of tetrahydrocannabinol, tetrahydrocannabinolic acid and cannabinol during their extraction from Cannabis sativa L. Am. J. Analyt. Chem. 70 (8), 920–925. https://doi.org/10.1134/S1061934815080183.

Wiboonsirikul, J., Kimura, Y., Kadota, M., Morita, H., Tsuno, T., Adachi, S., 2007. Properties of extracts from defatted rice bran by its subcritical water treatment. J. Agric. Food Chem. 55, 8759–8765. https://doi.org/10.1021/jf072041l.

Wiboonsirikul, J., Kimura, Y., Kanaya, Y., Tsuno, T., Adachi, S., 2008. Production and characterization of functional substances from a by-product of rice bran oil and protein production by a compressed hot water treatment. Biosci. Biotechnol. Biochem. 72, 384–392. https://doi.org/10.1271/bbb.70464.

Zengin, G., Menghini, L., Di Sotto, A., Mancinelli, R., Sisto, F., Carradori, S., Cesa, S., Fraschetti, C., Filippi, A., Angiolella, L., Locatelli, M., Mannina, L., Ingallina, C., Puca, V., D'Antonio, M., Grande, R., 2018. Chromatographic analyses, in vitro biological activities, and cytotoxicity of Cannabis sativa L. Essential Oil: a Multidisciplinary Study. Molecules 10 (23), 12. https://doi.org/10.3390/molecules23123266.