KAUNAS UNIVERSITY OF TECHNOLOGY

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Biorefining of Industrial Hemp (Cannabis Sativa L.) into Valuable Cannabinoid and Antioxidant Fractions by High Pressure Extraction Techniques

Master's Degree Final Project

Supervisor
Lect. Dr. Vaida Kitrytė

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Food Science and Safety (code 621E40001)

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" Biorefining of Industrial Hemp (*Cannabis sativa* L.) into Valuable Cannabinoid and Antioxidant Fractions by High Pressure Extraction Techniques"

DECLARATION OF ACADEMIC INTEGRITY

03 June 20 16
Kaunas

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SANTRAUKA

Sėjamoji kanapė (*Cannabis sativa* L.) auginama įvairiems tecnologiniams ir mitybiniams tikslams ir dėl joje esančių natūralių bioaktyvių junginių, tokių kaip kanabidiolis (CBD) ir canabidiolio rūgštis (CBDR) ir natūralių nekanabinoidinio tipo fenolinių antioksidantų, praktiškai pritaikomų funkcionaliųjų maisto komponentų, maisto papildų ir farmacijos pramonėje. Šio baigiamojo magistro projekto tikslas, pritaikyti kelių etapų ekstarkciją aukštu slėgiu siekiant išskirti kanabinoidų ir antioksidantų frakcijas iš pluoštinio tipo kanapių (*Cannabis sativa* L.) auginamų Lietuvoje.

Siekiant įgyvendinti šį tikslą buvo nustatytos optimalios superkrizinės ekstrakcijos anglies dvideginiu sąlygos (slėgis, laikas ir temperatūra). Įvertintas tikslinių kanabinoidų (CBD ir CBDR) kiekis ir *in vitro* antioksidacinės savybės, įvairiose *C. sativa* rūšių anatominėse dalyse prieš ir po SKE-CO_2 bei ekstraktuose. Tai atlikta siekiant nustatyti geriausią *C. sativa* rūšį tolimesniems biorafinavo etapams. Atsižvelgiat į gautus rezultatus buvo nustatyta, kad *C. sativa "Benico"* rūšis sukaupia didžiausią CBDR keikį, taip pritaikant SKE-CO_2 lipofilinėje frakcijoje galima sukoncentruoti 99 % CBD ir 88% CBDR, taip pat šios rūšies ekstrakta ir žaliava prieš ir po SKE-CO_2 pasižymėjo didžiausiu antioksidaciniu aktyvumu.

Keturių etapų biorafinavimas buvo naudojamas superkrizinės ekstrakcijos anglies dvideginiu ir slėginės ekstrakcijos parametrų (slėgio, temperatūros, laiko ir tirpiklio santykio) optimizavimui, siekiant gauti didžiausią ekstrakto bei bioaktiviųjų junginių kiekį. Optimizavus sąlygas, kanapių ekstarktuose buvo įvertintas kanabinoidų kiekis, *in vitro* antioksidacinis aktyvumas ir preliminari fitochemenimė sudėtis. Galiausiai, buvo nustatytas CBD ir CBDR toksiškumas ir poveikis CaCo-2 ląstelių pralaidumui, gauti rezultatai rodo, kad CBD, o ypač CBDR gali apsaugoti ląsteles nuo oksidacinio streso sukeltų pažeidimų.

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SUMMARY

Hemp (*Cannabis sativa* L.) is cultivated for various technological and dietary purposes and due to the presence of natural bioactive constituents, like cannabidiol (CBD) and cannabidiolic acid (CBDA) and natural non-cannabinoid type phenolic antioxidants has potential applications in functional food, pharmaceutical and nutraceutical industries. The aim of this work was to develop multistep biorefining protocol for valuable cannabinoid and antioxidant fraction isolation by high pressure extraction techniques from industrial (fiber-type) hemp (*Cannabis sativa* L.), grown in Lithuania.

To achieve this aim, optimal supercritical carbon dioxide extraction conditions (pressure, time and temperature) for lipophilic fraction isolation from various *C. sativa* cultivars, grown in Lithuania were determined. Target cannabinoid (CBD and CBDA) content and *in vitro* antioxidant properties of SCE-CO_2 extract and plant material prior and after SCE-CO_2 from various *C. sativa* cultivars of different vegetation periods and anatomical parts were assessed in order to select the most promising cultivar for further biorefining. The obtained results showed that *C.sativa* cultivar *'Benico'* containe the highest amount of CBDA, showed the highest efficiency of SCE-CO_2 to concentrate CBD (99%) and CBDA (88%) in lipophilic fraction, and distinguished by the highest antioxidant capacity for SCE-CO_2 extracts and plant material before and after extraction.

4-Step biorefining protocol for the cultivar *'Benico'* was developed by determining optimal supercritical carbon dioxide and pressurised liquid extraction parameters (pressure, temperature, time and solvent ratios) to obtain the highest extract and target bioactive constituent yields. Under optimized conditions, cannabinoid content, *in vitro* antioxidant potential and preliminary phytochemical composition of various isolated non-polar and polar *'Benico'* extracts was evaluated. Finally, the toxicity and effect on cell monolayer integrity of CBD and CBDA preparations was evaluated in Caco-2 cells, showing that CBD and mainly CBDA are efficient partially to correct oxidative stress-induced damages in cells.

LIST OF ABBREVIATIONS

$ABTS^{\bullet+}$	2.2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
CBD	Canabidiol
CBDA	Cannabidiolic acid
CCD	Central composite design
CO_2	Carbon dioxide
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl radical
EAE	Enzyme-assisted extraction
ETOH	Ethanol
GAE	Gallic acid
H_2O	Water
HPLC	High-performance liquid chromatography
ORAC	Oxygen radical absorbance
PLE	Pressurised liquid extraction
PRP	Phenol red passage
RSM	Response surface methodology
SCE-CO_2	Suppercritical carbon dioxide extraction
TEER	Transepithelial electrical resistance
TEAC	Trolox equivalent antioxidant capacity
TE	Trolox equivalents
TPC	Total phenolic content
UPLC	Ultra-performance liquid chromatography
WB	Western blotting

INTRODUCTION

Fiber-type hemp (*Cannabis sativa* L.) is one of the oldest plants in the world, cultivated mainly for oil, seed and fiber production. Today only cultivation and processing of *C. sativa* cultivars with low psychotropic cannabinoid THC content (<0.2 %) is officially legalized in various countries all over the world and from 2013 in Lithuania regulated by Order of the Minister of Agriculture of the Republic of Lithuania 3D-867 [1]. In addition to fiber-type hemp product utilization for food and industrial purposes, the presence of valuable bioactive constituents cannabinoids shows promising applications of hemp products in treatment of epilepsy, pain, depression and anorexia, cancer and other disorders. Furthermore, fiber-type hemps accumulate a considerable portion of non-cannabinoid type antioxidatively active phytochemicals, which quantitative and qualitative composition may vary in different *C. sativa* cultivars, plant vegetation periods, anatomical plant parts, growing, handling and storage after harvesting conditions. Due to these reasons various hemp fractions could be utilized as natural source of bioactive phytochemicals and antioxidants for plaussibe functional food, nutraceutical and pharmaceutical ingredient search and isolation. Therefore, the aim of this research – to develop multistep biorefining protocol for valuable cannabinoid and antioxidant fraction isolation by high pressure extraction techniques from industrial (fiber-type) hemp *(Cannabis Sativa* L.*)*, grown in Lithuania. To achieve this aim following objectives were raised:

1. To determine optimal supercritical carbon dioxide extraction conditions for lipophilic fraction isolation from various *C. sativa* cultivars, grown in Lithuania.
2. To determine yields, cannabinoid (CBD and CBDA) content and *in vitro* antioxidant properties of SCE-CO_2 extract and plant material prior and after SCE-CO_2 from various *C. sativa* cultivars of different vegetation periods and anatomical parts and to select the most promising cultivar for further biorefining.
3. To develop multistep biorefining protocol for the selected *C. sativa* cultivar by determining optimal supercritical carbon dioxide and pressurised liquid extraction parameters to obtain the highest extract and/or target bioactive constituent yields.
4. To evaluate yields, cannabinoid content, *in vitro* antioxidant potential and preliminary phytochemical composition of various isolated non-polar and polar extracts from the selected *C. sativa* cultivar and plant material prior and after each step of extraction under optimized conditions.
5. To evaluate major fiber-type hemp cannabinoids – CBD and CBDA – toxicity on Caco-2 cells and effect on cell monolayer integrity.

I. LITERATURE REVIEW

1.1. Morphology and chemical composition of Cannabis plants

Plants of genus *Cannabis,* belonging to family Cannabaceae, class Magnoliopsida, division Magnoliophyta and kingdom of flowering plants Plantae, are widely distributed all over the world. *Cannabis* were cultivated as crop plants for fiber, food oil and medicine production already from the Neolithic Era, predominately in Central Asia, with some evidences dating the 6th century B.C. [2, 3, 4]. According to the genetic, morphological and biochemical variations, today three gene pools of *Cannabis* plants are distinguished, namely *C. sativa*, *C. indica* and *C.ruderalis,* and the latest taxonomic circumscription thereof is as following[4, 5].

- *C. sativa* pool, containing hemp and feral biotypes. Hemp biotype comprises landraces from Europe, Asia Minor and central Asia, previously assigned to *C. sativa* L. subsp. *sativa* var. *sativa* accessions, grown for fiber and seed production. Typically, *Cannabis* plants cultivated for these purposes are referred to as hemp. To feral biotype, *C. sativa* L. subsp. *sativa* var. *spontanea* accessions, growing as ruderal populations in eastern Europe, are ascribed.
- *C. indica* pool, containing NLD (narrow-leaflet), WLD (wide-leaflet), hemp and feral biotypes. Both NLD and WLD are drug strains of *Cannabis*, previously assigned to *C. sativa* L. subsp. *indica* var. *indica* (Lam.*)* accessions from southern Asia, Africa and Latin America (NLD), Afghanistan and Pakistan (WLD). *C. indica* hemp biotype includes landraces from eastern and southern Asia, previously assigned to *C. chinensis* Delile accessions, while feral biotype – ruderal *C. sativa* L. subsp. *indica* var. *kafiristanica* accessions from India and Nepal.
- *C. ruderalis* pool, including ruderal populations from central Asia, previously assigned to *C. ruderalis* Janisch.

Looking at the average anatomical composition of these plants, stalks, roots, leaves and seeds comprise respectively 59%, 15%, 15% and 11% of total biomass weight, however with some variations in different *Cannabis* species, subspecies and varieties (**Fig. 1**). For example, *C. sativa* plants accumulates higher amount of stalks and total biomass as compared to the ones, belonging to *C. indica* gene pool. The latter plants distinguish by the highest density of leaves, while *C. ruderalis* accumulates low content of biomass, plant is short and leaves are thin. Typically, cultivars grown for fiber production distinguish by long not branched stems, while seed strains are often shorter and may be both, branched and not branched. Hemp biotypes are characterized by bigger seeds, as compared to plants of feral biotypes [6]. Different vegetation period causes

differences in chemical composition, variation on concentrations of constituents and visual appearance. Generally, hemp is maturated at *III* vegetation period and is accumulated the highest content of bioactive constituents (e.g. CBD, CBDA, THC) and biomass [7].

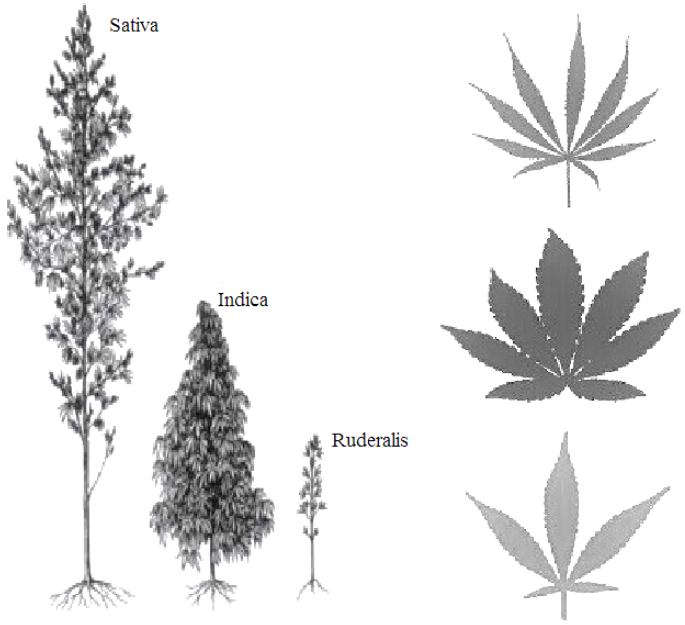


Fig. 1. Anatomical differences of plants from *C. sativa*, *C. indica* and *C. ruderalis* gene pools

The average quantitative and qualitative chemical composition of major nutrients in various *Cannabis* plants seeds is the following: carbohydrates (30%), lipids (25-35%), proteins (20-25%) [8]. Looking at the profile of carbohydrates (**Table 1**), various monosacharides (glucose, fructose, galactose, arabinose, mannose and rhamnose), disaccharides (sucrose, maltose) and polysaccharides (raffinose, cellulose, hemicellulose, pectin, xylan) have been identified in various plant anatomical parts [6]. The vast majority of carbohydrates are located in hemp stalk fibers, rich in cellulose (70%), hemicellulose (20%) and lignin (6%) [9]. Hemp seeds contain approximately 30% of the total carbohydrate content. Additionally carbohydrates are useful for dietary nutrition, in hemp seeds are 21.8% of digestible and 48.4% of non-digestible fibers [10, 11]. From nitrogen-containing constituents, several proteins (edestin, zeatin, zeatinnucleoside) and in total 18 amino acids (at the range of 0.2-3.1 % DW) were identified in *Cannabis* seeds (**Table 1**) and recognized as important constituents of human diet [11], all essential amino acids (lysine, methionine, threonine, tryptophan, histidine, isoleucine, leucine, valine) present in hemp *C. sativa.* Essential polyunsaturated fatty acids linoleic acid (C18:2, omega-6) and α-linolenic acid (omega-3), which play significant role in metabolism processes, protect cardiac cells against damages [12], and are proved to lower cholesterol levels and high blood pressure [13], are the major lipid constituents in *Cannabis* seed oil (**Table 1**). In addition to these fatty acids, polyunsaturated cis-vaccenic and iso-

linolenic acid, and several saturated acids (myristic, lignoceric, caproic, heptanoic, caprylic, pelargonic, capric, lauric, margaric, iso-arachidic) are present in lipophilic fractions [6].

Table 1. Average chemical composition of *Cannabis* plants

Compound	Amount	Compound	Amount
Carbohydrates, g/100g of cell wall components:			
Lignin	3.58	Galactose	3.78
Rhamnose	1.13	Glucose	83.43
Arabinose	1.95	Galacturonic acid	2.41
Xylose	1.71	Glucuronic acid	0.25
Mannose	5.13		
Fatty acids, g/100g of total lipids:			
Linoleic acid	53–60	Stearidonic acid	0.4– 2
α- linolenic acid	15–25	Eicosanoic acid	<0.5
Oleic acid	9–16	Palmitic acid	6–9
γ-linolenic acid	1–4	Stearic acid	2–4
Amino acids, g/100 g of total proteins:			
Alanine	1.28	Lysine*	1.03
Arginine	3.10	Methionine*	0.58
Aspartic acid	2.78	Phenylalanine	1.17
Cysteine	0.41	Proline	1.15
Glutamic acid	4.57	Serine	1.27
Glycine	1.14	Threonine*	0.88
Histidine*	0.71	Tryptophan*	0.20
Isoleucine*	0.98	Tyrosine	0.86
Leucine*	1.72	Valine*	1.28
Vitamins and minerals, mg/100 g:			
Vitamin E	90.0	Calcium (Ca)	145
Thiamine (B1)	0.4	Iron (Fe)	14
Riboflavin (B2)	0.1	Sodium (Na)	12
Phosphorous (P)	1160	Manganese (Mn)	7
Potassium (K)	859	Zinc (Zn)	7
Magnesium (Mg)	483	Copper (Cu)	2

*- essential amino acids

Minor compounds are found mainly in *Cannabis* seeds and leaves and include vitamins and minerals, amounting from 0.1 to 1160 mg/100 g (**Table 1**), phytosterols (campesterol, ergosterol, β-sitosterol, and stigmasterol), hydrocarbons (dimethyl alkanes, pentacosane, hexacosane, and hentriacontane, etc.), simple alcohols (methanol, ethanol), aldehydes (ethanol, acetaldehyde, isobutyraldehyde, pentanal), ketones, acids, esters, lactones more than 70 nitrogen-containing constituents (e.g. alkaloids: cannabisativine and anhydrocannabisativine, amides: grossamide and cannabisins, heterocyclic amines: piperidine, hordenine and pyrrolidine, and other: choline, trigonelline, muscarine, betaine, neurone) [14, 22]. Finally, *Cannabis* plants are natural sources of various bioactive secondary metabolites, such as terpenoids, polyphenols and more than 60 unique

terpenophenolics that haven't been detected in any other plant and are commonly referred to as cannabinoids [11, 15, 16]. As reported by De Meijer et al. (2003) [17] and more recently by Hilling and Mahlberg (2004) [5], amount of psychoactive cannabinoid tetrahydrocannabinol (THC) and its ratio to non-psychoactive cannabidiol (CBD) or its precursor cannabidiolic acid (CBDA) are very important taxonomic characteristics, differentiating *Cannabis* plants into three main chemotypes:

- Chemotype I: drug-type plants, accumulating 1–20% of THC, showing high total THC/CBD or THC/CBDA ratio (> 1) and thus affecting central nervous system;
- Chemotype II: intermediate-type plants with 0.3–1.0% of THC content, ratio ~1 THC/CBD or THC/CBDA ratio and no significant effects on central nervous system;
- Chemotype III: fiber-type plants, with <0.3% of THC, high amounts of CBD or CBDA (ratio < 1) and do not affecting central nervous system [5,18,17].

According to this classification, all *C. sativa* biotypes are characterized by low (<0.2 %) THC content and belongs to the chemotype III plants, while due to high amounts of THC accumulated *C. indica* strains are ascribed to the chemotype I plants [2].

1.2. Cultivation and industrial applications of fiber-type hemps

Today only cultivation and processing of fiber-type hemps from *C. sativa* gene pool with low THC content (<0.2 %) is permitted and officially legalized in Canada, USA (27 states), Europe (Austria, Denmark, Finland, Germany, Great Britain, Hungary, Italy, Poland, Portugal, Romania, Slovenia, Spain, Sweden, Switzerland, Turkey, Ukraine, Latvia, Lithuania and other countries), South America (Chile, Columbia), China, Egypt, India, Japan, Korea, the Netherlands, New Zealand, Russia and Thailand [19]. Growing of drug-type hemps and commercialization of their processing products is forbidden by European drug policy and Supreme Court of the United States. The content of THC is regulated by European Cities on Drug Policy and Drug Policy Alliance of United States. THC content is controlled as reported in EB regulation No.1122/2009 [20] and amending regulation No. 173/2011 [21] and identification of this compound is performed as reported in procedure "Recommended Methods for the Identification and Analysis of Cannabis and Cannabis Products, 2009" is prepared by Laboratory and Scientific Section United Nations Office on Drugs and Crime [22, 69, 70].

According to the FAO data of 2013, the major part (71%) of fiber-type hemp are grown in France, followed by the second biggest producer China with 25% of annual production. Numerous reports indicate that plant growing, climate, handling and storage conditions have significant impact on the *C .sativa* L. hemp biomass yield, as well as quantitative and qualitative composition

of target constituents in various anatomical plant parts. Industrial (fiber-type) hemps preferably grows in open and sunny places in well-drained nutrient-enriched soil with pH values of 7.0-7.5, while higher content of the muld in the soil causes lower yield of seeds and fiber [23]. About 42% of hemp biomass (mainly, leaves and roots) returns to the soil, providing nutrients for further crop growing. On average, hemp uptake 31% N, 67% P, 47% K, 28% Mg and 28% Ca from the soil, though the percentage of these minerals return to soil is higher: 69% N, 33% P, 53% K, 72% Mg and 72% Ca, this suggests, that hemp residues after harvesting could be utilized as a plant for the soil enrichment with nutrients [24]. During the period of vegetative growing and maturation, hemp requires 25-28°C temperatures [24]. In addition, hemp requires a lot of moisture 300 to 400 mm of rainfall. Drought during the period between flowering and seed maturation results in low yields of grain [7, 25, 26].

Due to the proper climate conditions, industrial hemps were widely cultivated in Lithuania as crop plants in the past. For example, *C. sativa* L. cultivation area in 1941 was approximately equal to 1500 hectares[25]. However, after the World War II fiber-type hemp cultivation and processing at industrial scale was forbidden for more than 60 years and legalized by the Ministry of Agriculture of the Republic of Lithuania only in 2013 [27]. Today all fiber-type hemp producers are controlled by Order of the minister of Agriculture of the Republic of Lithuania and have to render an account as referred in law regulation 3D-867 [1], approved by Order of the Minister of Agriculture of the Republic of Lithuania [1]. Currently one of the biggest hemp growing companies in Lithuania is JSC 'Agropro', cultivating more than 2500 ha annually. Taking into account smaller farmers (e.g. BPKAPA (Baltic fiber crops and Producers' Association), UAB „Šeimos šaknis) the total cultivation area of fiber-type hemps in Lithuania is much bigger and reaches more than 3000 ha. Some of well-known and widely cultivated *C. sativa* varieties in Europe as well as in Lithuania are the following: *'Finola'*, *'USO', 'Benico'*, *'Futura', 'Felina'*. *Finola'* is one of the recent cultivar cultivated for hemp seed oil production, firstly breeded by Dr. J.C. Callaway in 1997 in Finland and nowadays growing nearly in all Northern countries for food, oil or fiber production [28]. The maturity of this cultivar is one of the earliest, short stalks allow easy harvesting, the yield of seeds is higher as compared to other varieties, while seeds oil is characterized by an optimum balance of 18:3 omega-3 and 18:2 omega-6 fatty acids [25]. *'USO'* cultivar, characterized by early ripening and maturation and high amount of stalks and fiber, originally was cultivated in Ukraine. This cultivar was included in the EU catalogue in 1997 [25]. *Benico'* was first cultivated in Poland, plants are very high and accumulate higher yield of biomass. '*Benico'* is the most fibrous cultivar and also has the earliest ripening and maturation. Also this type of cultivar is suitable for food, oil, pharmacy production, but the biggest success it has in fiber

production [25, 29]. The plants of this cultivar produce more seeds and fiber comparing with other varieties [25].

The main current fiber-type hemp product application fields are schematically presented in **Fig. 2** Summarising, fiber-type hemp cultivates with low content of THC (<0.2%) can be grown as fiber, seed, or medicine crop for industrial product, food and pharmaceuticals production. For example, hemp stalks, containing cellulose (70%), hemicellulose (20%) and lignin (6%) [9], can be used to produce fibers, paper, home furnishings, carpeting, construction and isolation materials. Hemp seed oil is common ingredient in the cosmetics, personal body-care products and nutritional supplements. Seeds and pomace are utilized as food and as source for alternative food proteins, seeds are enriched with all essential fatty acids and easily digestible proteins [13, 30]. Additional application area is stalk biomass utilization for enhance fuels energy [30]. Nowadays only few companies producing hemp products are known in Lithuania, e.g. MB "Bioproduktas" (Hempus), MB Kanapis. However, currently hemp is one of the most promising plant and has big perspective in industry in the future.

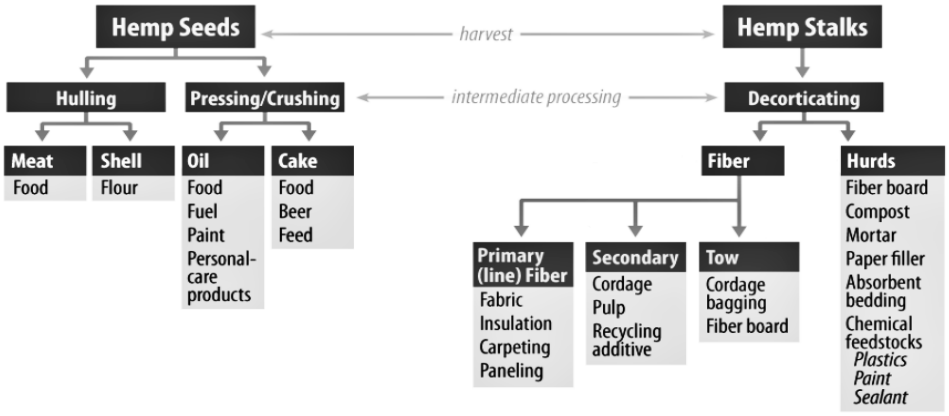


Fig. 2. Potential application areas of various fiber-type hemp possessing products and by-products [31]

In addition to the above discussed application areas, the interest towards the search of natural valuable bioactive hemp constituents with numerous bioactive properties *in vitro* and *in vivo* is obviously increasing [31]. It was shown that various anatomical parts of fiber-type hemp (mainly leaves and seeds) and their processing by-products could be utilized for cannabinoid and natural antioxidants isolation [3, 31] and thus provide promising future applications in functional food, pharmaceutical and nutraceutical industries [11].

1.3. Valuable bioactive constituents in fiber-type hemp

1.3.1. Cannabinoids

As introduced in Chapter 2.1, plants of genus *Cannabis* produces a unique class of terpenophenolic phytochemicals, called cannabinoids, which quantitative and qualitative composition differentiates *C. sativa* (fiber-type) and *C. indica* (drug-type) strains. The majority of these compounds are regarded as bioactive in various physiopathological processes, shown to modulate human endocannabinoid system, performing major regulatory homeostatic functions in digestive tract, liver, brain, cardiovascular, genitourinary and bone systems [32], and have important applications in pharmaceutical and nutraceutical industries [33]. During the flowering hemp accumulates the highest amount of biomass and chemical compounds [34]. Up to date, 66 cannabinoids were identified in *C. sativa* plants (**Table 2**), ascribed to the subclasses [6]:

- Cannabidiol (CBD)-type. In total, compounds were ascribed to this group with CBD and its parent compound cannabidiolic acid (CBDA) being the most abundant cannabinoids in fiber-type hemps;
- Cannabigerol (CBG)-type. CBG was the first identified cannabinoid. CBG and CBGA are the main representatives CBG-type class cannabinoids and are are starting compounds for other cannabinoids synthesis in hemps. Less abundant compounds are cannabigerolic adic monomethylether, cannabigerol monomethylethe, cannabigerovarin [5, 6].
- Δ^8-Tetrahydrocannabinol (Δ^8-THC) and Δ^9-tetrahydrocannabinol (Δ^9-THC)-types. Δ^9-THC is the main psychotropic compound in hemps, while Δ^8-THC is approximately 20% less active. As for CBD and CBG, the main precursor for Δ^9-THC is the non-psychoactive compound Δ^9-tetrahydrocannabinolic acid A (Δ^9-THCA-A). These compounds are present in hemp *C. sativa* in very low concentrations [6].
- Other types: cannabichromene (CBC), cannabinol (CBN), cannabinodiol (CBND) cannabicyclol (CBL), cannabielsoin (CBE) and cannabitriol (CBT). However, there is lack of studies about these constituents and their application is not very popular [6].

The qualitative and quantitative composition of various cannabinoids varies in fiber-type hemps and other Cannabis plants is presented in **Table 2**. The highest amounts of these compounds are generally accumulated in leaves and seeds, depending on plant vegetation period, environmental, growing and storage conditions [6]. Chemical structures of the main representatives from each above discussed cannabinoid class are depicted in **Fig. 3**.

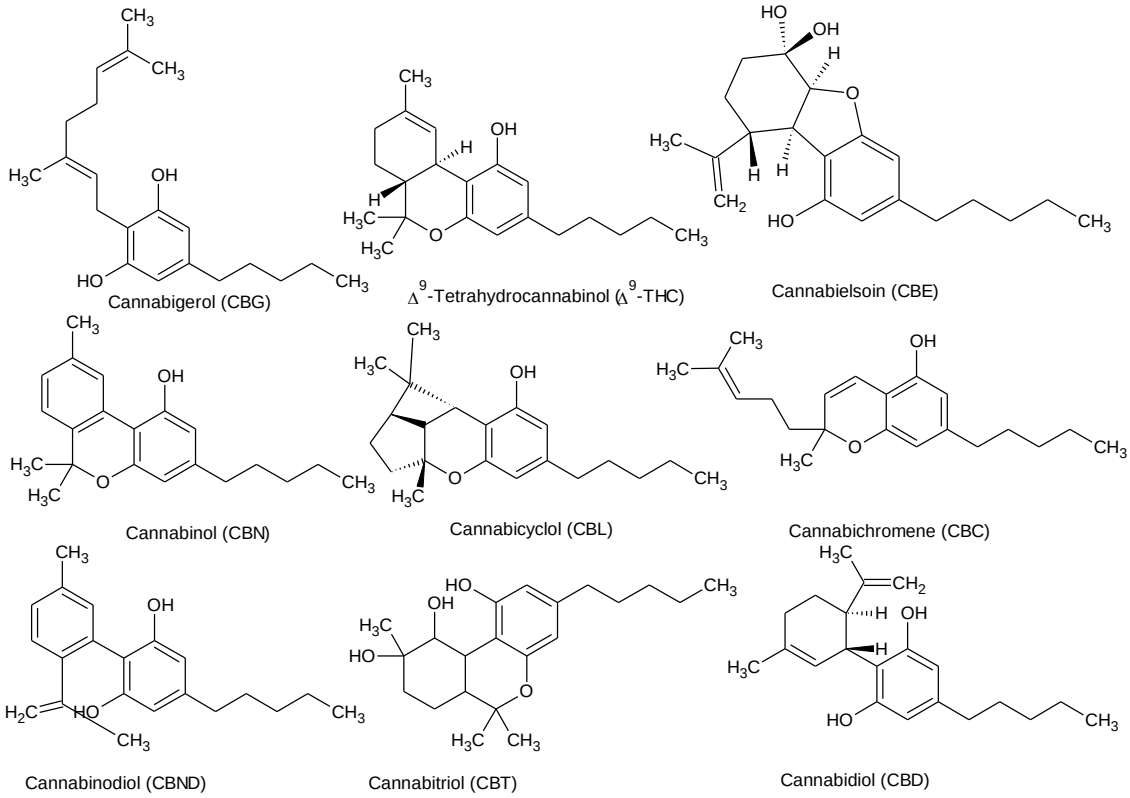


Fig. 3. Chemical structures of various cannabinoids in a hemp *C. sativa*

In *Cannabis* plants, cannabinoids are biosynthesized as their carboxylic acid derivatives and accumulated within glandular trichomes, which are located in aerial plant surfaces (mainly, flowering tops and bracts). Upon maturation, harvesting, heating, drying and storage of plant material, these carboxylic acids are further decarboxylated to neutral canabinnoids via the non-enzymatic thermal conversion pathway [4, 35]. As depicted in (**Fig. 4**), the main route for the major cannabinoid formation is via the degradation of CBGA and further decarboxylation, oxidation and isomerization reactions of various intermediate products formed. For example, THCA is obtained from CBGA through oxidocyclization reactions, catalysed by the plant endogenous enzyme THCA-synthase. THCA is further dexarboxylated to Δ^9- THC, which can be either isomerised to less active Δ^8-tetrahydrocannabinol (Δ^8-THC) or oxidized to CBN. The latter compound is regarded as THC by-product, since naturally does not occur in plants. CBGA is also precursor for CBCA and CBDA synthesis, which further may undergo decarboxylation to yield other important bioactive fiber-type cannabinoid CDB [8, 48].

Table 2. Classification and concentration of various cannabinoid classes in hemp *C. sativa* [6]

Class	Cannabinoids	Concentration, % DW
Cannabigerol	Cannabigerolic adic (CBGA); Cannabigerolic adic monomethylether (CBGAM); Cannabigerol (CBG); Cannabigerol monomethylethe (CBGM); Cannabigerovarinic adic (CBGA); Cannabigerovarin (CBGV)	0.03-1.15
Cannabichromene	Cannabichromenic acid (CBCA); Cannabichromene (CBC); Cannabichromevarinic acid (CBCVA); Cannabichromevarin (CBCV)	0.0-0.65
Cannabidiol	Cannabidiolic acid (CBDA); Cannabidiol (CBD); Cannabidiol monomethylether (CBDM); Cannabidiol C4 (CBD C4); Cannabidivarinic acid(CBDVA); Cannabidivarin (CBDV); Cannabidiorcol (CBD-C1)	0.1-2.89
Δ^9-Tetrahydrocannabinol	Δ^9-Tetrahydrocannabinolic acid A (THCA-A); Δ^9-Tetrahydrocannabinolic acid B (THCA-B); Δ^9-Tetrahydrocannabinol (THC); Δ^9-Tetrahydrocannabinolic acid C4 (THCA-C4); Δ^9-Tetrahydrocannabinol C4 (THC-C4)); Δ^9-Tetrahydrocannabivarinic acid (THCVA); Δ^9-Tetrahydrocannabivarin (THCV); Δ^9-Tetrahydrocannabinorcolic acid (THCA-C1); Δ^9-Tetrahydrocannabinorcol (THC-C1); Δ7-cis-iso-Tetrahydrocannabivarin	Fiber-type: < 0.2 Other types: 0.1-25
Δ^8-Tetrahydrocannabinol	Δ^8-Tetrahydrocannabinolic acid (Δ^8-THCA); Δ^8-Tetrahydrocannabinol (Δ^8-THC)	0.0-0.1
Cannabicyclol	Cannabicyclolic acid (CBLA); Cannabicyclol (CBL); Cannabicyclovarin (CBLV)	0.017
Cannabielsoin	Cannabielsoinic acid A (CBEA-A); Cannabielsoinic acid B (CBEA-B); Cannabielsoin (CBE)	0.003
Cannabinol and Cannabinodiol	Cannabinolic acid (CBNA); Cannabinol (CBN); Cannabinol methylether (CBNM); Cannabinol – C4 (CBN-C4); Cannabivarin (CBNV); Cannabinol – C2 (CBN-C2); Cannabiorcol (CBN-C1); Cannabinodiol (CBND); Cannabinodiovarin (CBVD)	0.1-1.6
Cannabitriol	Cannabitriol (CBT); 10-etoxy-9-hidroxy-delta-6a-tetrahydro Cannabitriol; 8,9-dihidroxy-delta-6a-tetarhydro Cannabitriol; Cannabitriovarin (CBTV); Etoxy- Cannabitriolvarin(CBTVE)	0.003
Others	Constituents with a furano ring (dehydrocannabifuran, cannabifuran), carbonyl function (cannabichromanon, 10-oxo-δ-6a-tetrahydrocannabinol), or tetrahydroxy substitution (cannabiripsol),	-

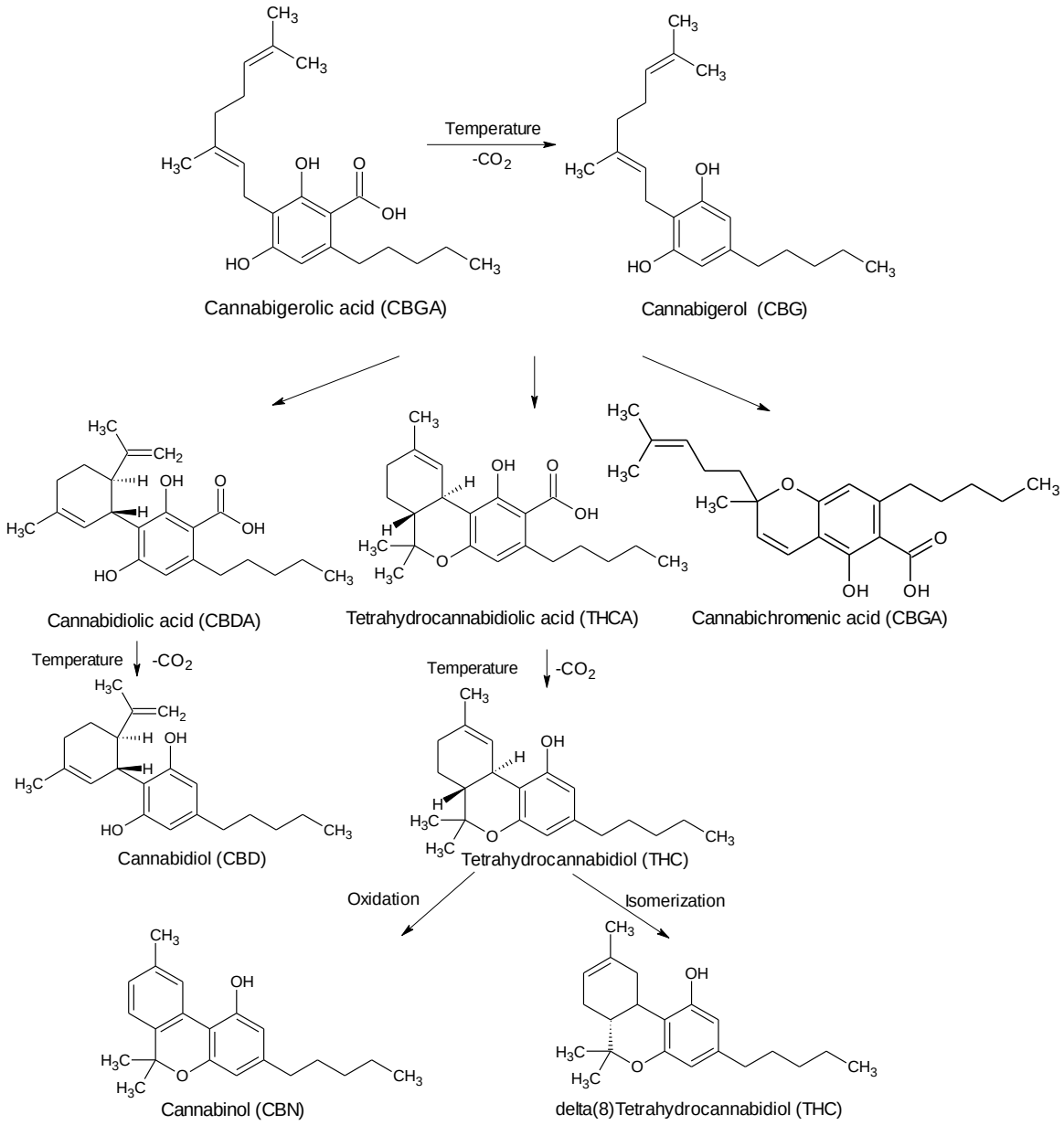


Fig. 4. Preliminary scheme of the main cannabinoids synthesis in *Cannabis* plants

As overviewed in **Table 3**, numerous up to date widely reported beneficial medical and therapeutic effects of cannabinoids are the following: analgesic (relieves pain) [36], anti-bacterial and anti-fungal [37], anti-diabetic (reduces blood sugar levels) [38], anti-depressant, anti-emetic (reduce vomiting and nausea) [39, 40, 41, 42], anti-epileptic (reduce seizures and convulsions), anti-inflammatory, anorectic (weight loss) [43], anti-insomnial (aids sleeping disorders), anti-ischemic (reduce risk of artery blockage), anti-proliferative (inhibits cancer cell growth) [44, 45, 46], anti-psioratic (effective in psoriasis treatments), anti-psychotic and tranquilizing [47, 48], ant-ispasmodic (suppresses muscle spasms), anxiolytic (relieves anxiety) [49, 50, 51], appetite stimulant [52, 53], bone stimulant (promotes bone growth) [54, 55, 56, 57, 58], intestinal anti-

prokinetic (reduces small intestine contractions) [59, 60, 61], neuroprotective (effective in retarding nervous system degeneration) [62, 63], vasorelaxant (reduces vascular tension) [64,65,66,67,68].

Table 3. Beneficial medicinal and therapeutic effects of various cannabinoids [33, 36, 37, 39, 40-51, 54-68]

Beneficial medical and therapeutic effects	**Cannabinoids**												
	CBD	**CBDA**	**Δ^9-THC**	**Δ^8-THC**	**CBG**	**CBGA**	**CBC**	**CBCA**	**THCA**	**THCV**	**THCVA**	**CBLA**	**CBNA**
Analgesic	+		+	+		+	+				+	+	+
Anorectic										+			
Anti-bacterial	+				+	+							
Anti-diabetic	+												
Anti-emetic	+		+										
Anti-epileptic	+									+			
Anti-fungal								+					
Anti-inflammatory	+	+				+	+	+					
Anti-insomnia					+				+				
Anti-ischemic	+												
Anti-proliferative	+	+			+		+		+				
Anti-psioratic	+												
Anti-psychotic	+												
Anti-spasmodic	+		+						+				
Anxiolytic	+												
Appetite stimulant			+										
Bone stimulant	+				+		+			+			
Intestinal anti-prokinetic	+												
Neuroprotective	+												
Vasorelaxant	+												

Looking at the activities of minor fiber-type hemp cannabinoids (**Table 3**), CBG and CBC-type compounds are characterized by antibiotic, anti-fungal, anti-inflammatory, analgesic properties [6, 47, 69]. Δ^9-THC and Δ^8-THC class derivatives are the main psychoactive and toxic hemp components, causing psychological and behavioural effects [6, 47]. However there are some indications that at low concentrations these cannabinoids could be potentially utilized for anti-emetic, anti-inflammatory, analgesic, antioxidant, euphoriant medicine production [6, 47]. CBN and CBND exert antioxidant, anti-microbial, anti-fungal, anti-inflammation properties, are effective against methicillin resistance *Staphylococcus aureus* microorganisms, decrease fat accumulation in the body, reduce nausea and decrease deleterious THC effects, thus are utilized for epilepsy, depression, seizures, psychosis, dependency and breast cancer treatments [70]. CBL demonstrates analgesic properties [6].

However, as reported by various researchers, non-toxic and non-psychotropic CBD and its parent compound CBDA are the most promising cannabinoids in plants from *C. sativa* gene pool with huge variety of beneficial pharmacological activities (**Table 3**): anti-biotic, anti-depressant, anti-fungal, anti-inflammatory, analgesic, antioxidant, anti-spasmodic, anti-psychotic [47, 71]. As shown by Alhamoruni and Wright (2012), CBD at 10 μM concentrations is able to modulate intestinal CaCo-2 cells membrane permeability, is important in epithelial barrier integrity and has promising applications in intestinal inflammatory treatments [72]. Additionally these observations were confirmed by Harvey (2014) and co-workers, who conducted experiments on CaCo-2 cells and evaluated CBD effect on bowel inflammatory, mucosal damages, colitis and prevention to oxidative stress [73]. In 2000 Chen and Buck [74] discovered that CBD protect cells from death and that CBD and to a greater extent CBDA inhibits vomiting in shrews and nausea in rats and could be utilized as a treatment for nausea and vomiting [40]. Also potential medical value in breast cancers treatment inhibiting migration of the invasive MDA-MB-231 human breast cancer cells was reported for CBDA at the concentrations of 10 and 25 μM [42, 46]. In addition to these activities, CBD contains a phenolic structure, which is typical of many antioxidants isolated from plants [74, 75]. The antioxidative properties of 10 μM CBD was confirmed by its ability to reduce oxidative stress and inhibit neuronal death by scavenging toxic reactive oxygen [65]. Cannabinoids are able to antagonize the oxidative stress which causes cells death and the suggested mode of action of CBD and CBDA is via the prevention of cells death by antioxidation [74].

Cannabinoids exert their well-known physiological effects through two G protein coupled cannabinoid receptors CB1 (first cloned in 1990) [76] and CB2 (first cloned in 1993) [77]. CB1 receptors modulate ion channels through direct G-protein interactions. CB2 is thought to function primarily in the immune central nervous system [78]. Cannabinoid receptors CB1 and CB2 are activated by endogenous agonists endocannabinoids (e.g. anandamide, 2-arachidonoylglycerol and others), endocannabinoids with cannabinoids receptors constitute endocannabinoid system, where endocannabinoids acts as reverse synaptic messengers and protect cells against damages [79, 80, 81, 82]. Activation of CB1 receptors reduces emesis, inhibits gastric acid secretion and intestinal motility, reduce spread of cancer, these and other effects that might be beneficial in the treatment of gastro diseases. Activation of CB2 receptors can inhibit immune cell migration and reduce clinical signs of inflammation and inflammatory bowel diseases and inhibits intestinal motility [83-92].

1.3.2. Non-cannabinoid type compounds

Besides very important phytocannabinoids, hemp is enriched with valuable non-cannabinoid type constituents (**Table 4**), such like: phenolic compounds, flavonoids, terpenoids, amino acids, fatty acids, carbohydrates and nitrogen containing constituents. Non-cannabinoid type phenolic compounds include phenolic acids and flavonoids, phenol methylethers, and phenolic glycosides. Non-cannabinoid phenolic compounds are identified as important bioactive constituents in fiber-type hemp. Some of them, e.g. cannabispiran, cannabistilbene, cannithrene, are unique for *C.sativa* plants and act as *in vitro* and *in vivo* antioxidants by reducing oxidation rates and protecting cells against damages [6, 93].

Table 4. Non-cannabinoid type bioactive constituents in fiber-type hemp

Compounds	Amount	Compound	Amount
Non-cannabinoid type phenols and flavonoids, % DW			
Ferulic acid	0.01	Cannabistilbene	0.00004
Cannabispiran	0.002-0.025	Cannithrene	-
Apigenin	0.1	Orientin	0.056
Luteolin	0.049	Isovitexin	0.009
Quercetin	0.01	Vitexin	0.029
Kaempferol	0.026	Cannflavins A/B	0.003-0.02
Terpenoids, % DW			
β- myrcene	0.47	d-limonene	0.14
β-caryophyllene	0.05	α-pinene	0.04

Flavonoids luteolin-7-O-glucoside, and apigenin-7-O-glucoside are the major flavonoids present in cannabis, while cannflavins A and B are unique for cannabis and accumulated in *C. sativa* cultivars on average at levels of 0.003-0.019%. As reported by Pietta (2000), that flavonoids act as anti-microbial agents as well as exert antioxidant, anti-allergenic, anti-viral and anti–inflammatory, anxiolytic, estrogenic, anti-mutagenic, anti-neoplastic activities in various *in vitro* and *in vivo* assays [15, 94,95, 96]. Flavonoid content from different *Cannabis* tissues is folloving: leaves > blossoms > seeds > roots [97]. Generally, cultivar has no big influence for flavonoids content in hemp [97, 98]. Chemical structures of the major non-cannabinoid type bioactive representatives are depicted in **Fig. 5**.

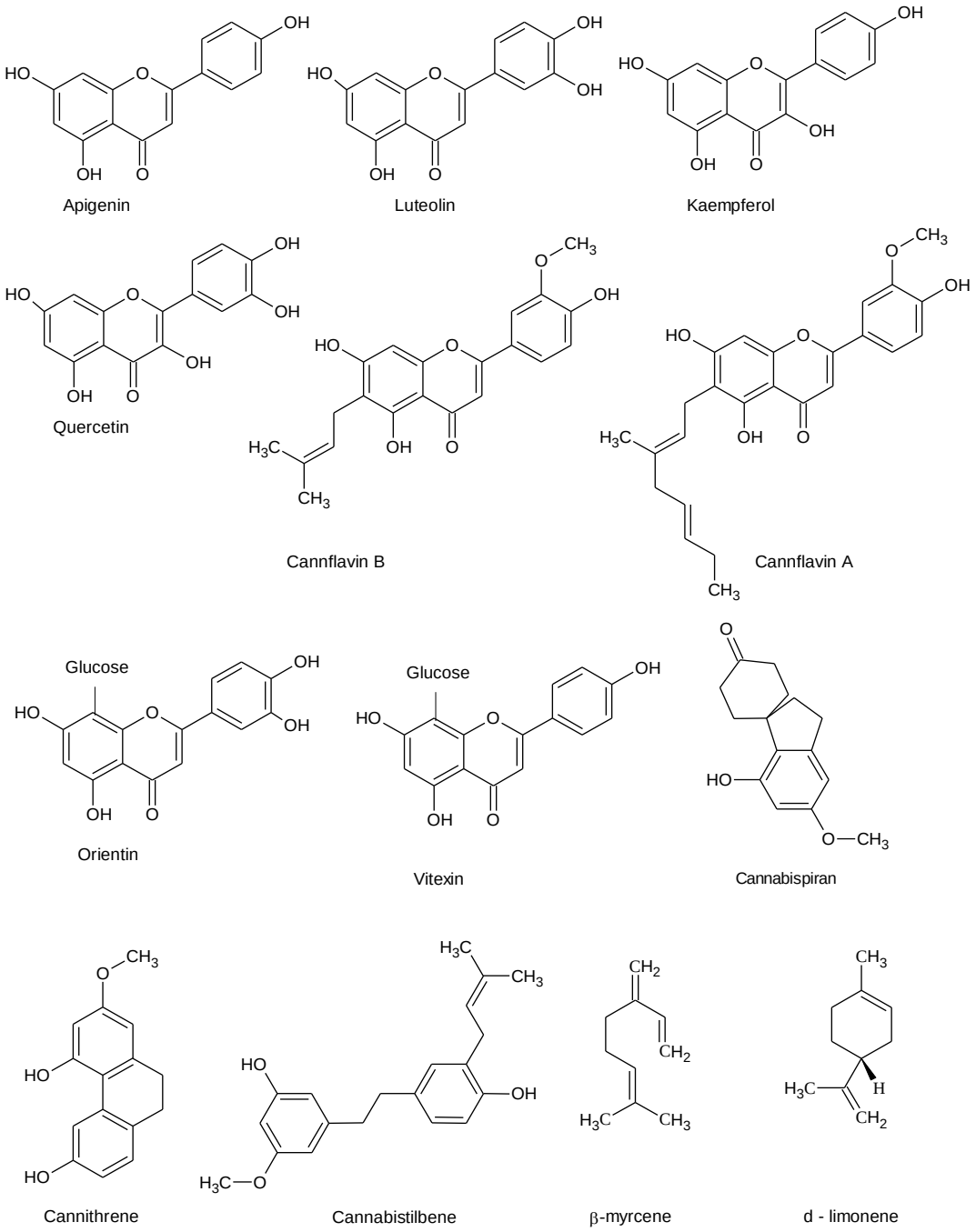


Fig. 5. Non–cannabinoid type flavonoids, phenols and terpenoids in hemp

Generally, terpenoids are accumulated in green leaves and flowers and extracted from hemp by steam distillation. Yields of obtained essential oil are in the range 0.05-0.11%. Terpenoids are responsible for the various flavours and are characterized by analgesic, anti-inflammatory, anti-biotic, anti-mutagenic, cytoprotective, anti-malarial, anti-depressant; memory booster, anti-pyretic, bronchodilator features [26, 99], can inhibit cholesterol synthesis, promote hepatic enzyme activity, and inhibit isoprenylation implicated in malignant deterioration of proteins [15]. Terpenoids composition depends on strains and harvest dates. [15] The main fiber-type hemp terpenoids are β-myrcene, β-Caryophyllene and d-limonene. The most abundant terpenoid β-

myrcene possess antibiotic potency against *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa*, and *Escherichia coli*, inhibits cytochrome P450 2B1, an enzyme involved in the metabolic activation of promutagens [15, 100, 101]. β-Caryophyllene is the most common sesquiterpenoid (the main constituent of copaiba anti-inflammatory balsam), clinically safe and useful as antimalarial agent [15, 102]. The second most common terpenoid in hemps is limonene – monocyclic monoterpenoid, commonly used in the perfumery and flavour industries. Limonene shows protective properties against aflatoxin induced cancer, inhibits the hepatic metabolism of the promutagen by inhibiting the growth of *Aspergillus fungi* and aflatoxins production [103, 104]. In addition, some of the above discussed compounds were reported to exert particular antioxidant capacity [63]. As was recently suggested for various cannabis extracts by Tomida and Pertwee, cannabinoids (e.g. CBD) have antioxidant properties and prevent neuronal death by scavenging toxic reactive oxygen [65]. Besides main antioxidativelly active lipophilic hemp seed oil compounds (β-carotene, lutein, vitamin A and and vitamin E), in 2011 Booz reported that CBD is more protective than α-tocopherol or vitamin C and hydroxytoluene, and unlike BHT does not promote growth of tumors [105]. Recently Chen and He (2012) studies reported about isolation and identification of two nitrogen-containing compounds – *N*-trans-caffeoyltyramine and cannabisin B (**Fig. 6**) – basically responsible for antiradical activity of hemp seed extract, isolated from *C. sativa* 'Bama' and 'Yunma' cultivars [8]. The IC_{50} value of *N*-trans-caffeoyltyramine (9.42 μg/mL) is lower than cannabisin B (11.17 μg/mL). Those constituents demonstrated strong activities of scavenging $DPPH^{\cdot}$ and protecting low-density lipoproteins against oxidation as compared to other plant extracts [8].

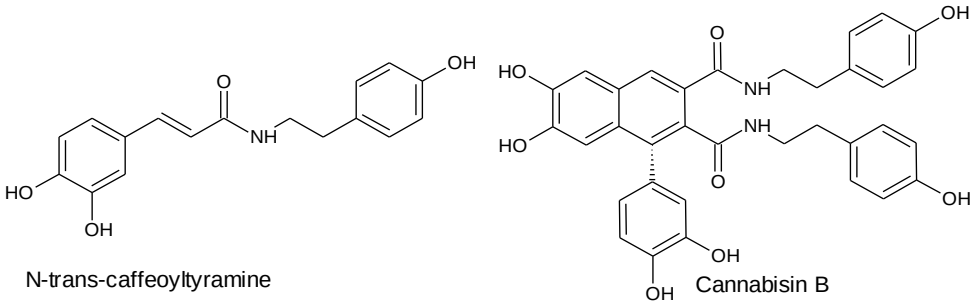


Fig. 6. *C.sativa* seed oil compounds with antiradical activity [8]

1.4. Conventional and innovative extraction techniques for isolation of bioactive fiber type hemp constituents

Over the last decade, a variety of conventional and innovative extraction and fractionation are available for lipophilic and hydrophilic constituent isolation from various fiber-type hemp cultivars and anatomical parts of plant (**Table 5**). Hemp seed oil enriched with cannabinoids,

unsaturated fatty acids and other bioactive components, is among the most valuable products of hemp. One of the most popular hemp seed oil extraction method is cold pressing. Although associated with best quality of oil, application of this technique results in low oil yield and high amount of by-products obtained (35%). Lyophilic fraction isolation efficiency can be increased utilizing different non-polar solvents, typically hexane, and petroleum ether. One of the classical solvent-based fractionation is Soxhlet extraction. This process is very efficient [8], and approximately 30% oil yield could be reached after 8 h of extraction at 70°C utilizing hexane as solvent and hemp seeds as starting material [13]. Other authors report that the major part of oil (~90%) is extracted within the first half an hour and later extraction rate significantly reduces [106]. Also, the oil yield remarkably increases up to 14 folds when samples of smaller particle size (≤0.2 mm) are utilized [106]. However the major problems for conventional solvent extraction are the following: (1) solvent removal and problematic polar and bound lipids elimination from the sample; (2) long extraction time; (3) high percentage of toxic residues (e.g. limits of hexane 290 ppm, acetone 30 ppm) [13, 107]; (4) low extraction efficiency of target metabolites for some solvents and plausible degradation of valuable constituents upon solvent removal procedure. In 2013 Romano and Hazekamp [108] reported that the content of cannabinoids and terpenoids is considerably lower in petroleum ether extracts as compared to other solvents utilized (e.g. CO_2, ethanol, acetone) with particular losses observed comparing qualitative composition of β-myrcene, β-caryophyllene, β-pinene [108, 109].

To solve these issues, supercritical carbon dioxide extraction (SCE-CO_2) became important alternative technique to isolate lipophilic constituents from various plants [13, 110]. Although being more expensive, SCE-CO_2 is more efficient as compared to conventional solvent-extraction based methods, since provides the ability to isolate and concentrate high spectrum of the plant valuable components without using organic solvents [111]. This extraction technique is popular due to the main advantages, such as lower extraction temperatures applied and choice of odourless, non-toxic and non-flammable solvents (e.g. CO_2), which can be easily removed from the extracted oil at the end of the process by lowering its pressure and allowing the gas to quickly and completely dissipate, while elimination of ethanol costs at least 0.5 eur/1 kg plant material [112, 113, 114].

Table 5. Conventional and innovative extraction techniques of *C. sativa*

Extraction type	Anatomical part	Cultivar	Extraction parameters				Yield, %	Reference
			Time	Particle size, mm	Temperature, °C	Pressure, bar		
Conventional extraction techniques:								
Water	Blossoms	Felina	3 h	0.2-0.6	100	-	0.24	Da Porto and Decorti (2014) [115]
Ethanol	Seeds	Felina	-	~0.2	-	-	9.0	Lesma and Consonni (2014) [116]
Ethanol	Seeds	Futura	-	~0.2	-	-	7.8	Lesma and Consonni (2014) [116]
n-hexane	Seeds	-	8 h	-	25	-	31.48	Uluata and Ozdemir (2012) [117]
n-hexane	Seeds	Felina	8 h	1.5	70	-	30.6	Da Porto and Decorti (2012) [13]
n-hexane	Seeds	Felina	8 h	0.83	25	-	30.0	Da Porto and Natolino (2013)[118]
n-hexane	Seeds	-	15 h	0.7	25		41	Tomita and Machmudah (2013) [28]
Heptane	Seeds	Felina	8 h	~0.2	25	-	26.2	Lesma and Consonni (2014) [116]
Heptane	Seeds	Futura	8 h	~0.2	25	-	29.6	Lesma and Consonni (2014) [116]
Inovative extraction techniques:								
SCE-CO_2	Seeds	-	180 min	0.7	80	400	44.2	Tomita and Machmudah (2013) [28]
SCE-CO_2	Seeds	Felina	60 min	0.71	40	300	21.05	Da Porto and Voinovich (2012)[119]
SCE-CO_2	Seeds	Fedora	2.5 h	0.38	40	400	33.3	Aladic and Jarni (2015) [120]
SCE-CO_2	Blossoms	Felina	-	0.2-0.6	40	100	1.03	Da Porto and Decorti (2014) [115]
SCE-CO_2	Seeds	Felina	-	1.5	40	300	22.1	Da Porto and Decorti (2012) [13]
SCE-CO_2	Seeds	Felina	-	1.5	80	400	22.1	Da Porto and Decorti (2012) [13]
SCE-CO_2	Seeds	Felina	-	1.5	80	300	16.8	Da Porto and Decorti (2012) [13]
SCE-CO_2	Seeds	Felina	30 min	0.83	40	300	21.20	Da Porto and Natolino (2013)[118]
Ultrasound (40KHz)+Water	Kernel	Bama, Yunma	30 min	-	25	-	14.37	Chen and He (2012) [8]
Ultrasound (40KHz)+EtOH 50%	Kernel	Bama Yunma	30 min	-	25	-	10.02	Chen and He (2012) [8]
Ultrasound (40KHz)+MeOH 50%	Kernel	Bama, Yunma	30 min	-	25	-	8.23	Chen and He (2012) [8]
Ultrasound (40KHz)+Acetone 50%	Kernel	Bama, Yunma	30 min	-	25	-	8.44	Chen and He (2012) [8]
Ultrasound (20KHz)+ SCE-CO_2	Seeds	Felina	10 min	0.83	25	-	24.50	Da Porto and Natolino (2013) [118]

As reviewed by Herrero et al. (2010), CO_2 can be utilized to isolate easily-oxidized and heat-sensitive constituents without any toxic organic solvent, thus is friendly for nature and humans, does not pollute environment, can reduce amount of wastes generated, lead to higher efficiency and selectivity and yields high purity products for successful exploration in food and pharmaceutical industries [13, 121]. Due to the different nature of various plant materials, extraction temperature, time, pressure and particle size are the main process variables that should be optimized for efficient SCE-CO_2 [52]. In **Fig. 7** , the general schematic representation of SCE-CO_2 process and equipment is depicted. Shortly, liquid CO_2 released from vessel runs through a compressor and heater to increase the pressure and temperature higher than its critical point and to reach supercritical conditions (74 bar, 32°C) [122] [123]. Into expansion vessel, solvent and solute streams runs into each other and solute solubility is increased by higher pressure applied [112]. A supercritical liquid is characterized as high density liquid-like gas [112, 124], which solubility and absorption capacity increases with higher density and pressure. The gases are characterized by high diffusion and low viscosity, that causes high mass transfer rates between a solute and a supercritical liquid [125]. Carbon dioxide as a solvent is used due to higher density, solubility and lower critical parameters comparing with other supercritical liquids (e.g. water (373°C, 220 bar), ethanol (240°C, 61 bar), acetone (234°C, 47 bar)). The energy costs for extraction are lower than with other fluids. It is easy to get pure and not expensive CO_2. Therefore, nowadays supercritical CO_2 is regarded as the cheapest from the point of solvent price and solvent elimination and the most promising supercritical solvent for industrial applications [112, 126].

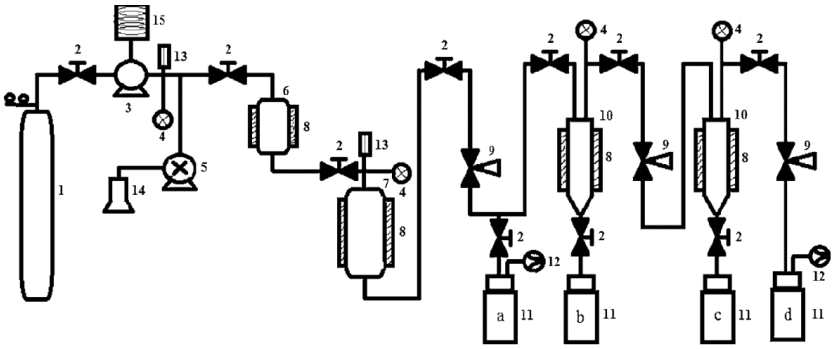


Fig. 7. Schematic representation of SCE-CO_2 process and equipment
1 – CO_2 cylinder; 2 – on-off valve; 3 – CO_2 pump; 4 – pressure gauge; 5 – cosolvent pump; 6 – CO_2 vessel; 7 – extraction vessel; 8 – heat jacket; 9 – manual back pressure regulator; 10 – separator; 11 – collection vials (a, b, c, d); 12 – gas flow metre; 13 – safety valve; 14 – cosolvent; 15 – CO_2 pump chiller (adapted from [127])

The application of SCE-CO_2 was previously demonstrated by various research groups (**Table 5**) as promising separation and fractionation technique to obtain hemp extracts enriched with cannabinoids and antioxidants. In addition, it was shown that SCE-CO_2 derived hem oil has

lower amounts of free fatty acids, directly indicating lower oxidation level as compared to solvent-extracted oil (e.g. acetone, ethanol, hexane) [128, 127]. In other study of Porto and Decorti (2012), differences in isolated oil of hemp seeds by hexane in Soxhlet and SCE-CO_2 were reported (30 and 22 %, respectively), however yielded oil by SCE-CO_2 under optimal conditions (300 bar, 40°C, 4 hours) was characterized by significantly higher oxidative stability, close to values obtained for virgin olive oil [13].

As reviewed in **Table 5**, SCE-CO_2 combined to ultrasonic-treatment could be utilized for lipophilic fraction and bioactive substances extraction from hemp seeds too. The ultrasonic treatment enhance particles disruption of cell walls, particle size reducing and cavitation increases mass transfer of the cell content [129]. The disintegration of acoustic cavitation bubbles provokes temporary spots with high pressure and temperature and the effect of ultrasound depends on many factors, like ultrasound frequency, pressure, temperature and sonication time [118, 130]. In 2013 Porto and Natolino extracted hemp seeds, employing different fractionation methods – Soxhlet extraction with hexane, SCE-CO_2 and ultrasound-assisted extraction – and further compared antiradical capacity and fatty acids composition in obtained lipophilic extracts. Authors reported that SCE-CO_2 yielded 21 g of oil per 100 g of seeds, which exhibited the highest antiradical capacity and oxidative stability as compared to other treatments: SCE-CO_2 > Ultrasound (20 Hz, 10 min, 25°C) > Soxhlet [118]. Combined SCE-CO_2 and ultrasound pre-treatment improved oil yield only by 3.3 g/100 g, did not alter fatty acids composition and desirable ω-6 to ω-3 ratio (3:1), and ~2-fold decreased original antiradical capacity as compared to SCE-CO_2 alone [118]. Prolonged ultrasound treatment for 20 and 40 min prior SCE-CO_2 slightly increased antiradical properties, which could be attributed to the sonication-induced formation of endogenous antioxidants in seeds [131, 132], however longer ultrasound treatment, significantly decreased oil yield (by 7.5 g/100 g DW). The hexane-extracted oil yield was the highest and amounted ~ 30 g/100 seeds, however significant decrease in antioxidant power was reported and could be explained by a partial degradation of the original antioxidant compounds (mainly, tocopherols) of hemp seeds.

Looking at the target bioactive constituents, cannabinoids are mainly isolated and concentrated in lipophilic fraction with a yield ~90%. Thus SCE-CO_2 could be efficient for cannabinoid isolation too [133]. Hemp terpenoids are typically isolated by hydrodistillation, which is common widely applied technique for aromatic compounds and essential oil isolation from plants. Particle size is one the most important parameters to optimize essential oil isolation by hydrodistiliation [98]. However as reported in Da Porto and Decorti studies, SCE-CO_2 under optimal conditions (100 bar, 40 °C) can yield 1.03% of essential oil, which is significantly higher

in comparison to amount obtained after 3 hours of hydrodistilation (0.24%). These observations are also confirmed by Rossi and ElSohly (2014), who reported similar essential oil yield of 0.29% by hydrodistilation [16]. Application of different extraction techniques may induce variations in quantitative composition of major volatile constituents are observed too. For example, Da Porto and Decorti reported that content of α-pinene, β-pinene and myrcene is approximately 11-52% higher in SCE-CO_2 extract, while hydrodistilation product is rich in terpinolene, caryophyllene, α-humulene and caryophyllene oxide (up to 2 fold higher quantities) [115]. Therefore, on the basis of reported literature data SCE-CO_2 technique is one of the most promising techniques for hemp oil, enriched with natural antioxidants, cannabinoids and particular terpenes, isolation.

Other very interesting and promising constituents of fiber-type hemp and non-cannabinoid type phenolic compounds (phenolic acids, flavonoids), which are concentrated in hydrophilic fraction and exert numerous bioactive properties. In most of the reviewed studies, various polar solvents, preferably water, ethanol, methanol and acetone and are used for hydrophilic fraction isolation and valuable phytochemical fractionation (**Table 5**) [109, 134]. According to the literature data, ethanol is commonly mixed with water to reduce swelling of the plant particles and to increase the permeability of the cell walls and enhance diffusion of extracted substances into solvent [135]. Studies performed by Chen and He (2012) indicated that ultrasonic extraction with 10 different polar solvents obtained by mixing ethanol, acetone and methanol with water under different ratios (0, 50, 75, 100%) yielded respectively 1.5-10.0 %, 0.5-8.4% and 5.7-8.2% of antioxidatively active polar extracts from hemp seeds, which activity increased in the following order: ethanol > acetone > methanol [8]. As reported by Lone and Lone (2012), 0.96% of water-soluble constituents were obtained from hemp leaves after 5 hours of extraction, while acetone under the same conditions extracted 0.64%. These researchers also revealed that protein content in acetone extract is up to 2.6 fold higher as compared to aqueous extract [37, 134]. Pressurised (accelerated) solvent extraction (PLE) is the alternative technique for different hydrophilic compounds isolation that is compatible to various solvents (methanol, acetone, ethanol, water and other) to quickly extract phenolic acids, flavonoids and polyphenols from plant samples. PLE is operated at temperatures above solvents boiling temperature and pressure of 10.3 MPa is used to keep the solvents in liquid form [136]. The main requirements for PLE solvent selection: high selectivity for the target constituents; high capacity for extraction; easily evaporative and cheap; not dangerous for environment and human [135].

In addition to the above discussed extraction and fractionation techniques, enzyme-assisted extraction has gained a lot of interest for bioactive constituent isolation over the last decade too [137]. The use of enzymes can lead to recovery of wide spectrum of industrial products and food

ingredients from hemp biomass. Non-starch polysaccharides, such as arabinoxylans and other pentosans, causes high viscosity of substances, which limits process efficiency, increases water consumption and energy supply for transportation. Non-starch polysaccharides has negative impact for processing, reduce efficiency of separation, evaporation, and heat exchange [138]. In addition, various commercially available cellulolytic (e.g., Viscozyme ®L) and other enzymes allows selective extraction of valuable nutrients (e.g. sugars, proteins), essential oils and phenolics. In most of plants phenolic compounds are present as soluble and insoluble forms (covalently bounded to cell wall structural or sugar parts) [139]. Kroon, Faulds, Ryden, Robertson, and Williamson (1997) [140] proposed that only small content of ferulic acid (2.6%) was available in gastric and small intestinal and in the colon (during fermentation) over 95% of feruloyl groups from wheat fiber were released [140]. This study suggests that bioavailability of bounded phenolic compounds is lower as compared to unbounded phenolic compounds. EAE may increase overall phenolic compound yield via cell-wall breakdown and enzymatic cleavage of glycosilated conjugates thereof [139, 141, 142].

1.5. Characterisation of bioactive fiber type hemp constituents

Various hemp fractions, obtained by above discussed conventional and innovative extraction techniques (Chapter 2.4), can be further analysed and characterized typically determining qualitative and quantitative composition of cannabinoids and other phytochemicals, assessing *in vitro* antioxidant capacity and evaluating *in vivo* bioactive properties.

For CBD and CBDA identification and quantification, it is recommended to follow the procedure of the protocol "*Recommended Methods for the Identification and Analysis of Cannabis and Cannabis Products*", prepared in 2009 by Laboratory and Scientific Section of United Nations Office on Drugs and Crime. This procedure includes all stages, starting from sample preparation to the final high-performance liquid chromatography HPLC analysis, which is officially approved for the most important cannabinoids (THC, CBD and CBDA) content determination in various *Cannabis* samples [22] and also used for phytochemical characterization of hemp extracts and fractions thereof [32, 133, 143, 144,145]. Generally, HPLC coupled to various detectors (MS, UV, DAD, NMR, GC–MS analysis) is one the most precise method for various components identification offering high reproducibility, good linear range and automation. Phytochemical composition could be also analysed by various ultra-performance liquid chromatography (UPLC), offering higher resolution, speed, and sensitivity as compared to HPLC [146, 147].

Another important characteristic of various plant extracts is *in vitro* and *in vivo* antioxidative properties thereof. Antioxidants are used as ingredients in food industry as agents to prolong shelf-

time of food products [148]. According to the model of action, antioxidants are classified into two groups: (1) primary – chain-breaking antioxidants, which react with free radicals, donate electrons and transform them into more stable products; (2) secondary, which stop the chain reactions via elimination of reactive oxygen [149]. From the technological point of view, the ability to control oxidation reactions (mainly, lipid oxidation) in foods is one of the most challenging tasks for food technologists and food safety specialists. Typically this is implemented via the addition of synthetic (e.g. butyl hydroxyl anisole, butyl hydroxyrotoluene, propyl gallate, tertiary butyl hydroquinone, etc). However their safety is highly question nowadays, thus demand for search and utilization of natural antioxidants for food, pharmaceutical and nutraceutical purposes is obviously increasing. In addition, natural plant antioxidants are important in cancer and coronary heart disease prevention [148], since inhibits oxygen, peroxides, or free radicals induced oxidation reactions and have protecting efect on cells from oxidative stress, cells death and various damages [149].

The main alimentary sources of natural antioxidants are plants (fruits, vegetables, spices, medicinal herbs, cereals, etc), their products and processing by-products [136, 150]. Those antioxidants include: (1) minerals (these are co-factor of antioxidants enzymes, their deficiency affect metabolism); (2) vitamins (e.g., vitamin C, vitamin E) (3) phytochemicals with phenolc structures (e.g., phenolic acids, flavonoids, cannabinoids, etc.) [149]. Qualitative and quantitative antioxidants evaluation aspect is very important for food scientists and technologists for both scholar and industrial applications. There are variety of direct and indirect colorimetric (e.g. Folin-Ciocalteu's, DPPH, ABTS, FRAP), fluorometric (e.g. ORAC), chromatographic (e.g. On-line DPPH, on-line DPPH assays) and mainly spectrophotometric (e.g. TRAP) methods, designed to determine *in vitro* antioxidant capacity of plant material [151, 152, 153]. As suggested by Prior et al., for representative evaluation of *in vitro* antioxidant potential of plant extracts it is recommended to measure oxygen radical scavenging properties by means of ORAC assay, TPC by Folin-Ciocalteu's assay and to employ several electron or hydrogen transfer based assays, e.g., $DPPH^{\bullet}$, $ABTS^{\bullet+}$ or FRAP [151, 154, 155]. Nevertheless, the total antioxidant capacity of plant materials may be still highly underestimated due to differences in extraction procedures applied [156] and incomplete recovery (extraction) of bioactive constituents [139, 157], which may remain bound to cell wall polysaccharides [158]. Thus the solid residues after the extraction still may be a potential source of beneficial phytochemicals, as was recently showed by our research group for amaranth seeds [106], brewery spent grain [155], raspberry pomaces [159]. To overcome these disadvantages and evaluate the total antioxidant capacity of foods, Serpen and co-workers (2007) recently suggested a novel procedure – QUENCHER approach. This technique is compatible with

all common antioxidant capacity assays and does not require extraction step prior to measurements, since simultaneously soluble part participates in the typical liquid-liquid interactions and the activity of insoluble part is measured due to the surface reactions at the solid-liquid interface [160, 151]. Since there is a lack of data on overall hemp plant material and its various extract antioxidant activity, QUENCHER approach could be successfully expored for these purposes too.

In addition, various *in vivo, in vitro* and *exvivo* bioassays are very important for evaluation of biological, pharmacological activity, side effect (toxicity) and physical effects of substances on living cells [161, 162]. Some of these bioassays show the effect of treatment on the expression or localization of proteins on cells (e.g Western blotting, immunofluorescence), correlating to the inflammation (biological body response to damages; enhanced expression of proteins) level and presence of damages in cell cultures [66]. Correct localization of proteins means that cells are in normal condition (not damaged), while outspreaded proteins indicate damages on cells, those damages can be visualised with specific confocal microscope [163]. Therefore, application of these assays to evaluate CBD and CBDA toxicity and effect on cell monolayer integrity would provide additional information about these cannabinoid properties.

II. MATERIALS AND METHODS

2.1. Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH•, free radical, 95%), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX, 97%), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), Folin & Ciocalteu's phenol reagent (2M), gallic acid (99%), microcrystalline cellulose (20 μm) and Viscozyme®L (cellulolytic enzyme mixture, ≥100 FBGU/g) were obtained from Sigma-Aldrich (Bornem, Belgium); $FeCl_3 \cdot 6H_2O$ (>99%) and sodium acetate (>99%) from Acros Organics (Geel, Belgium); 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) and fluorescein (FL) from Fluka Analytical (Bornem, Belgium); 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diamonium salt (ABTS), NaCl, KCl, Na_2HPO_4 and $K_2S_2O_8$ were from Merck (Darmstadt, Germany); KH_2PO_4 was from Jansen Chimica (Beerse, Belgium); Na_2CO_3 (98%, anhydrous) from RPL (Grauwmeen, Belgium). Standard compounds CBD (99.9%, 1 mg/ml methanol) and CBDA (99.9%, 1 mg/ml methanol) were obtained from THC Pharm GmbH (Frankfurt/Main, Germany), D-(+)-glucose (>99%), Cannabinoid preparations containing 100% of CBD and 27% of CBDA were provided by JSC 'Plantex Biotech' (Kaunas, Lithuania). Food grade ethanol was obtained from JSC 'Stumbras' (Kaunas, Lithuania), carbon dioxide (99.9%) from AGA (Vilnius, Lithuania), solvents were of analytical and HPLC-grade. The human intestinal Caco-2 cell line from Euroclone (Milan, Italy). Dulbecco's modified minimum essential medium DMEM; 3.7 g/L $NaHCO_3$, 4 mM/L glutamine, 10% heat inactivated fetal calf serum, 1% non-essential amino acids, 10^5 U/L penicillin and 100 mg/L streptomycin from Biochrom (Milan, Italy). Radioimmune protein (RIPA: 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Na deoxycholate, 1% Triton X- 100) buffer supplemented with 1 mM phenylmethylsulphonyl fluoride, Complete Mini (protease inhibitor cocktail) from Roche (Milan, Italy) and PhosSTOP (phosphatase inhibitor cocktail) from Roche (Milan, Italy). BSA bovine serum albumin from Zymed Laboratories (Thermo Fisher Scientific) (Waltham, MO USA). PBS without calcium chloride and magnesium chloride, 10×, liquid, sterile-filtered, suitable for cell culture from Sigma-Aldrich (St. Louis, MO, USA). PBS with calcium chloride and magnesium chloride, 10×, liquid, sterile-filtered, suitable for cell culture from Sigma (St. Louis, MO, USA). Antibodies cannabinoid receptors CB1 (PA1-745) and CB2 (PA1-746A) were from Thermo Fisher Scientific (Waltham, MO USA), occludin, β-catenin, NF-κB, p- NF-κB, β-tubulin were from Zymed Laboratories (Thermo Fisher Scientific) (Waltham, MO USA). Transwell filters, polyethylene terephthalate filter inserts for cell culture, 3.0-mm pore diameter were from Becton Dickinson (New Jersey, JAV). Fluorescein isothiocyanate (FITC) or tetramethyl rhodamine

isothiocyanate (TRITC) were from Jackson Immuno Research Labs (West Grove, USA). Sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 g/L bromophenol blue, 10 mM b-mercaptoethanol), TBS (50 mM Trizma base, 500 mM NaCl), TTBS (50 mM Trizma base, 500 mM NaCl, 0.05% Tween-20) were from Sigma-Aldrich (St. Louis, MO, USA). Nitrocellulose sheets from Schleicher and Schuell, Bioscience (Allentown, USA). Chemiluminescence reagent from Euroclone LiteAblot Extend (Milan, Italy). Prolong Gold antifade Reagent from Molecular Probes, Invitro- gen (Milan, Italy).

2.2. Plant material

Dried plants of *C. sativa* varieties *'Finola'* and *'USO'* at different vegetation periods, dried mixture of *C. sativa* blossoms, leaves and stems (variety not specified by the provider) and *C. sativa* processing by-products, containing different amounts of seed hulls, were provided by JSC 'Agropro' (Vilnius, Lithuania). Dried mixture of seeds, blossoms and leaves from *C. sativa* variety *'Benico'* **Table 6**. Plant samples were kept in a dry, well-ventilated and dark place and prior to the extraction were ground by ultra centrifugal mill ZM 200 (Retsch, Haan, Germany) using 0.2 mm hole size sieve.

Table 6. *C.sativa* samples used in this study

Plant material	**Vegetation period**		
	I	II	III
'Finola'			
Seeds and blossoms	+	+	+
Leaves	+	+	+
Stems	+	+	+
Roots	+	+	+
'USO'			
Seeds and blossoms	-[n]	-[n]	+
Leaves	-[n]	-[n]	+
Stems	-[n]	-[n]	+
Roots	-[n]	-[n]	+
'Benico'			
Mixture of seeds, blossoms and leaves	-[n]	-[n]	+
C.sativa* , variety unspecified:			
Mixture of blossoms, leaves and stems	-[n]	-[n]	+
C.sativa*, by-products I*			
Seed hulls	-[n]	-[n]	+
C.sativa, by-products II***			
Seed hulls	-[n]	-[n]	+

-[n]: not available; *: plant material used for SCE-CO_2 optimization; **: seed hulls (major part); ***: seed hulls (minor part)

2.3. Supercritical CO_2 extraction (SCE-CO_2)

SCE-CO_2 was performed in a supercritical fluid extractor Helix (Applied Separation, Allentown, PA) as described by Kraujalis and Venskutonis (2013) [164], with slight modifications. Each extraction was carried out using 10 g of ground sample loaded into a 50 mL stainless steel extraction vessel (inner diameter – 14 mm, length – 320 mm) between two layers of cotton wool in both ends to avoid particle clogging in the system. The temperature of the extraction vessel was controlled by the surrounding heating cover. The extracts were collected in glass bottles. The volume of CO_2 consumed was measured by a ball float rotameter and digital mass flow meter in standard liters per minute (SL/min) at standard state (P_{CO2} = 100 kPa, T_{CO2} = 20°C, ρ_{CO2} = 0.0018 g/mL). The flow rate of CO_2 in the system was controlled manually by the micro-metering vale (back-pressure regulator) and kept constant during all experiments at 2-3 SL/min. The schematic representation of SCE-CO_2 equipment is depicted in **Fig. 7.** For the SCE-CO_2 optimization, the mixture of C.*sativa* seeds, blossoms, leaves and stems (variety not specified) was used. The conditions for the extraction were set as follows: extraction pressure 75-475 bar, temperature 35-70°C, time 60-120 min (**Table 7**). In order to optimize SCE-CO_2 parameters for isolation of lipophilic constituents from a mixture of *'Benico'* seeds, blossoms and leaves, the extraction were set as follows: extraction pressure 100-500 bar, temperature 35-70°C, time 60-120 min (**Table 7**). A static time of 10 min was included in to the total extraction time and was constant in all extractions. The amount of extracts was determined gravimetrically (±0.001 g). The solid residue after the SCE-CO_2 was collected and kept in a dry, well-ventilated and dark place prior to the analysis. All the experiments were performed in duplicates.

Table 7. Levels of independent variables

Experimental factors	Variable levels					
	C.*sativa, variety not specified*			'*Benico'*		
	-1	*0*	*+1*	*-1*	*0*	*+1*
Extraction pressure (P, bar)	75	275	475	100	300	500
Extraction temperature (T, °C)	35	52.5	70	35	52.5	70
Extraction time (τ, min)	60	90	120	60	90	120

2.4. Pressurised liquid extraction (PLE)

PLE was performed in an accelerated solvent extraction apparatus ASE 350 (Dionex Sunnyvale, CA, USA) from residue of *'Benico'* seeds, blossoms and leaves after optimized SCE-CO_2 utilizing solvents of different polarity (acetone and mixtures of ethanol/water at different ratios), as previously described by Kraujalis (2013) [165] with some modifications. 5 g of ground plant material were mixed with 5 g of diatomaceous earth (1:1) and placed in a 34 mL Dionex

stainless-steel extraction cells (2.9 cm diameter, equipped with a stainless steel frit and a cellulose filter at the ends of the cell to avoid solid particles in the collection vial). To optimize extraction parameters, PLE with acetone was conducted at the following conditions: 30, 70, 100 and 130°C temperature; 15 min (3 cycles × 5 min), 45 min (3 cycles × 15 min), 75 min (3 cycles × 25 min) static extraction time. PLE with ethanol was conducted the following parameters: 1/4, 1/1 and 4/1 ethanol/water ratio (v/v), 100°C temperature, 45 min static extraction time (3 cycles × 15 min). The system pressure (10.3 MPa), pre-heating time (5 min), cell flush volume (100%) and purge time (120 s) with nitrogen to collect the extract in the vial were kept constant for all PLE experiments. The organic solvents were evaporated in a Büchi V–850 Rotavapor R–210 (Flawil, Switzerland), residual water was freeze-dried (-50°C, 0.5 mbar), extracts were kept in a freezer (-20℃) prior to the analysis, amounts were determined gravimetrically (±0.001 g).

2.5. Enzyme-assisted extraction (EAE)

The EAE was carried out using cellulolytic enzyme mixture Viscozyme® L by modified procedure of Kapasakalidis et al. (2009) [166]. 10 g of '*Benico*' sample after PLE-EtOH/H_2O (4/1, v/v) were weighted in a 250 mL polyethylene flat-bottom centrifugation bottle, suspended in 100 mL of 50 mM sodium acetate buffer (pH 3.5), followed by the addition of Viscozyme® L to reach the E/S ratio of 6% v/w (corresponds to 72 FBGU/10 g plant material). Appropriate control samples *Blank A (sample +buffer)*, *Blank B* (*enzyme+buffer*), and *Blank C* (*buffer*) were prepared simultaneously. Prepared mixtures were incubated in thermostatically controlled shaker (800 rpm) at 40°C for 7 hours. EAE was terminated by immersing centrifugation bottle in a boiling water bath for 10 min, followed by the rapid cooling and centrifugation (9000 rpm, 10 min). Resulting supernatants (water-soluble fractions) and solid residues (water-non soluble fractions) were collected, freeze-dried and kept in a freezer (-20°*C*) prior to the analysis. The amount of water-soluble fraction was determined gravimetrically (±0.001 g).

2.6. Cannabinoid analysis by HPLC-DAD

Quantitative determination of cannabidiol (CBD) and cannabidiolic acid (CBDA) in SCE-CO_2 extracts and plant material before and after SCE-CO_2 of various *C.sativa* samples (**Table 1**) was performed by the procedure of United Nations recommendations ST/NAR/40 "Recommended Methods For The Identification And Analysis Of Cannabis products" (United Nations, New York, 2009) [22]. To 500 mg of ground sample 5 mL of methanol: chloroform (90:10, v/v %) mixture was added, followed by 10 s of vortexing, 15 min of extraction in uultasonic bath (including again vortexing after 5, 10 and 15 min), and centrifugation (3000 rpm 5 min). Prior to the analysis, to

100 μL of separated optically clear supernatant 900 μL of HPLC-grade methanol was added. SCE-CO_2 extracts were prepared in methanol at the final concentration of 1 mg/mL. The analysis of target cannabinoids was performed on Simadzu HPLC chromatographic system, equipped with SIL 30AC automatic injector, CTO-20AC thermostat, DGU 20A5 vacuum degasator, LC-30AD pump and SPD-M20A diodes matrix detector and Supelco Discovery HS reversed-phase C18 (25×4.6 mm, 5 μm) column with C18 pre-column at the following conditions: thermostat temperature 30°C; isocratic conditions using mobile phase of acetonitrile/water (FA 0.1%) (4:1, v/v); run time 30 min; eluent flow rate 0.8 mL/min; injection volume 20 μL; detection at 225 nm (CBD) and 306 nm (CBDA). Data were processed by using Lab Solution program. The external calibration curves (peak area versus injected amount) of standard compounds CBD and CBDA were used for quantification.

2.7. Sugar analysis by UPLC–MS

The content of monosaccharide glucose and disaccharide maltose in EAE-derived supernatant and corresponding control sample *Blank A* (*sample +buffer*) from '*Benico*' sample was determined on an Acquity UPLC H-class system, equipped with a quaternary solvent delivery system and an autosampler. A BEH Amide column (1.7 μm, 100 × 2.1 mm, i.d.) was used for separation of compounds at 35°C. The samples (concentration 1 mg/mL, injection volume 1μL) were analysed under isocratic conditions using mobile phase consisting of 75/25% CH_3CN/ultra-pure H_2O with 0.1% NH_4OH at a flow rate of 0.35 mL/min. MassLynx 4.1 software was used for instrument control and data acquisition. MS experiments were performed in negative ionization mode, the capillary voltage was maintained at 2.8 kV, cone voltage at 25V, source offset at 50 V. Dessolvation gas (nitrogen) temperature was 350°C and flow rate 800 L/h, cone gas flow was 150 L/h, nebuliser pressure was 7.0 Bar. Chromatograms were recorded in SIR mode, using 178.8 m/z for glucose (dwell time 0.12 s) and 341.0 m/z for maltose (dwell time 0.2 s). Peak identification was carried out by comparing the retention times with those of the corresponding standards. The external calibration curves (peak area versus injected amount) of standard compounds (glucose for monosacharides and maltose for dissacharides) were used for quantification.

2.8. Phytochemical characterisation by UPLC/ESI–QTOF–MS

Phytochemical composition of '*Benico*' SCE-CO_2, PLE-Acetone, PLE-EtOH/H_2O extracts, EAE-derived supernatant and corresponding water-soluble fraction from control sample *Blank A* (*sample +buffer*) was screened on an Acquity UPLC system (Waters, Milford, USA) equipped

with a Bruker maXis UHR-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), binary solvent delivery system, an autosampler with a 10 μL sample loop, column manager, photodiode array (PDA) detector and an Acquity BEH C18 column (1.7 μm, 50 x 2.1 mm, i.d.), as previously described by Kraujalytė, Venskutonis, Pukalskas, Česonienė and Daubaras (2013) [147] with following modifications. The mobile phase initially consisted of eluent A (1% v/v formic acid in ultra-pure water), followed by an increase from 0% to 100% of eluent B (acetonitrile) over 9 min. During the following 2 min, the amount of eluent B was maintained at 100% for 1 min, followed by the re-introduced initial conditions over 1 min and the equilibration time of 1 min. Separation of compounds was performed at 25°C; the column was equilibrated for 1 min before each run; the flow rate 0.4 mL/min; extract concentration 1 mg/mL; injection volume 1μL. The effluent (monitored at 254 nm) from the PDA detector was introduced directly into the UHR-TOF mass spectrometer equipped with an ESI source. MS data were recorded in ESI negative ionisation mode. The capillary voltage was maintained at +4000 V with the end plate offset at –500 V. Nitrogen was used as the drying and nebulizing gas at a flow rate of 10.0 L/min and a pressure of 2.0 bar. For the instrument control and data acquisition, the Compass 1.3 (HyStar 3.2 SR2) software was used. Preliminary peak identification was carried out by comparing accurate masses of compounds with hose reported in literature sources and free chemical databases (Chemspider).

2.9. *In vitro* antioxidant activity assessment of *C. sativa* extracts and insoluble fractions

2.9.1. Sample preparation

For the *in vitro* antioxidant activity measurements in Folin-Ciocalteu's, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, FRAP and ORAC assays, various *C.sativa* extracts were dissolved in methanol (SCE-CO_2 and PLE-EtOH/H_2O) and acetone (PLE-Acetone) and further diluted with methanol to a final concentration from 5 to 1000 μg/mL. EAE-derived supernatant and corresponding water-soluble fraction from control sample *Blank A* (*sample +buffer*) were dissolved in dist.H2O to a final concentration from 500 to 4000 μg/mL. Antioxidant properties of insoluble fractions were evaluated applying QUENCHER approach (Gökmen, Serpen & Fogliano, 2009) [167]. Samples from the different steps of *C.sativa* biorefining process (before and after SCE-CO_2, PLE and EAE) were prepared following Serpen et al. (2007) [160] with some modifications. Separate stock mixtures of ground plant material (0.2 mm) were prepared in microcrystalline cellulose at a concentration of 500 μg/mg. Prior to the analysis, a series of "solid dilutions" of stock mixture with microcrystalline cellulose were performed to a final concentration from 2 to 100 μg/mg. All absorbances were measured with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic,

Rochester, NY) and antioxidant capacity was expressed as Trolox equivalent antioxidant capacity (TEAC, mg TE/g sample), unless indicated differently.

2.9.2. Total phenolic content (TPC) by Folin-Ciocalteu's assay

TPC of *C.sativa* extracts and EAE-serived water-soluble fractions was evaluated by the modified procedure of Singleton, Orthofer and Lamuela-Raventós (1999) [168]. 300 μL of sample (250-4000 μg/mL) or distilled H_2O (blank) were mixed with 1500 μL of Folin-Ciocalteu's reagent (1:9, v/v) and 1200 μL of Na_2CO_3 solution (75 g/L), left in dark for 2 hours and absorbance was measured at 760 nm. For the QUENCHER procedure, 10 mg of sample (15-100 μg/mg) or cellulose (blank) were mixed with 150 μL of distilled H_2O, 750 μL of Folin-Ciocalteu's reagent, and 600 μL of Na_2CO_3 solution, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, centrifuged (4500 rpm, 5 min) and the absorbance of optically clear supernatant was measured at 760 nm. The TPC was expressed as Gallic acid equivalents (mg GAE/g sample) by means of dose-response curves for Gallic acid [0-80 μg/mL; extract: $y=0.0114x-0.0559$ ($R^2=0.9926$); QUENCHER: $y=0.0114x+0.0128$ ($R^2=0.996$)].

2.9.3. The DPPH$^{\bullet}$ scavenging assay

The DPPH$^{\bullet}$ assay was carried out by the modified procedure of Brand-Williams, Cuvelier and Berset (1995) [169]. To a 2000 μL of a ~89.7 μmol/L (final absorption adjusted to 0.800±0.010 AU at 517 nm) DPPH$^{\bullet}$ methanolic solution 1000 μL of extracts (30-4000 μg/mL) or MeOH (blank) were added, mixtures were left in dark and absorbance was measured after 2 hours at 517 nm. For the QUENCHER approach, 10 mg of sample (15-60 μg/mg) or cellulose were transferred to a centrifugation tube, mixed with 500 μL of MeOH and 1000 μL of a ~89.7 μmol/L DPPH$^{\bullet}$ methanolic solution, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, centrifuged (4500 rpm, 5 min) and the absorbance of optically clear supernatant was measured at 517 nm. $TEAC_{DPPH}$ was calculated by means of dose-response curves for Trolox [0-50 μmol/L MeOH; extract: $y=1.5581x+0.5709$ ($R^2=0.9976$); QUENCHER: $y=1.445x+1.0315$ ($R^2=0.986$)].

2.9.4. The ABTS$^{\bullet+}$ scavenging assay

Following the protocol of Re et al. (1999) [170], firstly, phosphate buffer saline (PBS) solution (75 mmol/L; pH 7.4) was prepared by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 L of ultra-pure water. The ABTS$^{\bullet+}$ solution was prepared by mixing 50 mL of ABTS (2 mmol/L PBS) with 200 μL $K_2S_2O_8$ (80 mmol/L) and allowing the mixture to stand in the dark at room temperature for 15-16 h before use. The working solution was prepared by diluting the ABTS$^{\bullet+}$ solution with PBS to obtain the absorbance of AU 0.700±0.010 at 734 nm.

To a 3000 µL working $ABTS^{\bullet+}$ solution 50 µL of extract (20-4000 µg/mL) or MeOH (blank) were added, mixtures left in the dark for 2 hours and absorbance measured at 734 nm. For the QUENCHER approach, 10 mg of sample (1-25 µg/mg) or cellulose were transferred to a centrifugation tube, mixed with 25 µL of MeOH and 1500 µL of working $ABTS^{\bullet+}$ solution, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, centrifuged (3000 rpm 5 min) and the absorbance of optically clear supernatant was measured at 734 nm. $TEAC_{ABTS}$ was calculated by means of dose-response curves for Trolox [0-1500 µmol/L MeOH; extract: y=0.0467x-5.1121 (R^2=0.9959); QUENCHER: y=0.0536x+2.6694 (R^2=0.996)].

2.9.5. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out by the method of Benzie and Strain (1996) [171] with some modifications. For the analysis, 100 µL of extract (250-4000 µg/mL) or MeOH (blank) were mixed with 300 µL of distilled water and 3000 µL pf freshly prepared FRAP reagent [10 mmol/L TPTZ (in 40 mmol/L HCl), 20 mmol/L Fe $Cl_3 \cdot 6H_2O$, and 0.3 mmol/L sodium acetate buffer (pH 3.6), in ratio 1:1:10]. Mixtures left in the dark for 2 hours and absorbance measured at 593 nm. For the QUENCHER approach, 10 mg of sample (6-25 µg/mg) or cellulose were transferred to a centrifugation tube, mixed with 50 µL of MeOH, 150 µL of distillated water and 1500 µL of freshly prepared FRAP reagent, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, centrifuged (3000 rpm 5 min) and the absorbance of optically clear supernatant was measured at 593 nm. $TEAC_{FRAP}$ was calculated by means of dose-response curves for Trolox [0-500 µmol/L MeOH; extract: y=0.001x-0.0174 (R^2=0.991); QUENCHER: y=0.0012x-0.0097 (R^2=0.9984)].

2.9.6. Oxygen radical absorbance capacity (ORAC) assay

ORAC was evaluated by procedure of Prior et al. (2003) [172] using fluorescein as a fluorescent probe and 96-well black opaque microplates. 25 µL of extract (5-1000 µg/mL) or distilled H_2O (blank) were mixed with 150 µL of fluorescein solution (14 µmol/L PBS), preincubated for 15 min at 37°C, followed by a rapid addition of 25 µL of AAPH solution (240 mmol/L PBS) as a peroxyl radical generator using multichannel pipette. For the QUENCHER approach, 10 mg of sample (2-25 µg/mg) or cellulose (blank) were transferred to a centrifugation tube, mixed with 150 µL of PBS solution (75 mmol/L) and 900 µL of fluorescein solution, vortexed for 15 s, shaken at 250 rpm for 30 min in the dark and centrifuged (3000 rpm 5 min). Optically clear supernatant (175 µL) was transferred to the 96-well black opaque microplates, preincubated for 15 min at 37°C, followed by a rapid addition of 25 µL of AAPH solution. The fluorescence was recorded every cycle (1 min x 1.1), total 150 cycles using 485-P

excitation and 520-P emission filters in the FLUOstar Omega reader (BMG Labtech, Offenburg, Germany).

Raw data were exported from the Mars software to Excel 2003 (Microsoft, Roselle, IL) and the area under the fluorescence decay curve (AUC) was calculated from the normalised curves (fluorescence versus time) as:

$$AUC = 1 + \sum_{i=1}^{i=150} \frac{f_i}{f_0}$$

Where: f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i*.

$TEAC_{ORAC}$ was calculated by means of dose-response curves for Trolox [0-500 μmol/L MeOH; extract: y=0.1022x+9.512 (R^2=0.9928); QUENCHER: y=0.1041x+7.1582 (R^2=0.9930)].

2.10. CBD and CBDA preparations' activity assessment in Caco-2 cells

2.10.1. Epithelial cell culture preparation

Human intestinal Caco-2 cells were routinely grown on plastic tissue culture flasks (75 cm^2 growth area, Becton Dickinson, Milan, Italy) in Dulbecco's modified minimum essential medium (DMEM, 3.7 g/L $NaHCO_3$, 4 mM glutamine, 10% heat inactivated fetal calf serum, 1% non-essential amino acids, 10^5 U/L penicillin and 100 mg/L streptomycin) and maintained at 37°C in an atmosphere of air / CO_2 (95/5, %) at 90% relative humidity, as previously described by Finamore et al [173]. For transepithelial electrical resistance **(**TEER), phenol red passage (PRP) and immunofluorescence measurements, Caco-2 cells were seeded in multi-wells on Transwell filters (1.5×10^5 cells), grown in DMEM and maintained for 17 days to allow differentiation. Medium was changed three times per week. Prior to measurements, DMEM medium was changed to antibiotic- and serum-free DMEM medium. For Western blotting (WB) assay, CaCo-2 cells were grown in multi-wells without Transwell filters under the above described conditions.

2.10.2. Membrane permeability by transepithelial electrical resistance (TEER) assay

TEER measurements were conducted in accordance to the protocol of Ferruzza et al. [174, 175]. Caco-2 cells were subjected to AAPH (25 mg/ml), CBD (100%) and CBDA (27%) preparations (1-10 μM) and combined AAPH/cannabinoid treatment, and TEER was monitored every 60 min using Millicell Electrical Resistance System (Millipore) with STX2 electrode, one set of electrodes was placed in the basolateral compartment and the other in the apical compartment. TEER values of samples was measured at the onset of the experiment DMEM medium was changed to antibiotic- and serum-free DMEM. TEER values were expressed Ω/cm^2. Experiments were performed in duplicate.

2.10.3. Membrane permeability by phenol red passage (PRP) assay

Phenol red passage (PRP) was measured according to Ismail (1991) [176] and Ferruzza et al. (2002) [177], with slight modifications. Briefly, following three washes of cell monolayers with PBS (with calcium chloride and magnesium chloride) 0.5 mL of 1 mM phenol red was added in the apical compartment, whereas 1 mL of PBS was added in the basolateral compartment. After 1 h of incubation at 37 °C, 0.9 mL of basolateral medium was collected, treated with 0.1 ml of 0.1 N NaOH and absorbance was read at 560 nm to determine the phenol red concentration using TECAN Infinite M200 spectrophotometer (Männedorf, Switzerland) The passage of phenol red was expressed as apparent permeability [Papp, (cm s^{-1})×10^{-6}] and obtained from the following equation:

$$P_{app} = C_t \times \frac{V_{BL}}{\Delta t} \times C_0 \times A$$

where: V_{BL} is the volume of the basolateral compartment (cm^3), A is the filter area (cm^2), Δt is time interval (s), C_t is the phenol red concentration in the basolateral compartment at the end of time interval; C_0 is the phenol red concentration in the apical compartment at time zero.

2.10.4. Protein expression by Western blotting assay

Western blotting (WB) assay was performed following the procedure of Roselli et al. [178], with slight modifications. Firstly, Caco-2 cells were subjected to AAPH (25 mg/ml), CBD (100%) and CBDA (27%) preparations (1-10 μM) and combined AAPH/cannabinoid treatment, and TEER was monitored every 60 min as described in Section 2.10.2. After 6 hours of treatment, Caco-2 cells were lysed in cold radioimmune protein (RIPA) buffer, supplemented with 1 mM phenylmethylsulphonyl fluoride, protease inhibitor cocktail and phosphatase inhibitor cocktail. For the analysis, 150 μL of RIPA were added to the cells, incubated for 30 min in ice-bath, homogenized and centrifugated at 11000 rpm for 30 min at 4°C. After centrifugation, tubes were gently removed and supernatant was transferred in a fresh tube kept in ice-bath prior to the analysis. 15 μL of supernatant were mixed with 10 μL of sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 g/L bromophenol blue, 10 mM b-mercaptoethanol) and 15 μL of dist.H_2O, boiled for 5 min, centrifuged and electrophoretically transferred and fractionated by SDS-polyacrylamide gel (4–20% precast polyacrylamide gel) (Schleicher and Schuell, Bioscience, Allentown, USA). The proteins were transferred onto 0.2 mm nitrocellulose membrane according to the manufacturer's instructions (Trans-Blot Turbo Biorad, Milan). Membranes were incubate for 1 hour at room temperature (~24°C) with blocking solution [5% BSA in TBS (50 mM Trizma base, 500 mM NaCl)], washed with TTBS solution (50 mM Trizma base, 500 mM NaCl, 0.05% Tween-20), and incubated on shaker over the night (at least for 12 hour) at 4°C temperature with the following primary antibody (CB1, CB2, occludin, β-catenin, NF-κB, p- NF-κB, β-tubulin) at

the final concentration of 1 μL/mL 3% BSA in TBS. After incubation with primary antibody, membrane was washed with TTBS and incubated with HRP (horseradish peroxidase) conjugated secondary antibodies (anti-rabbit or anti-mouse IgG) diluted at the final concentration of 0.2 μL/mL in TTBS at room temperature (~24°C) for 1 hour on the shaker. Proteins were detected with enhanced chemiluminescence reagent (ECL kit LiteAblot Extend, Euroclone), followed by analysis of chemiluminescence with the charge-coupled device camera detection system Las4000 Image Quant (GE Health Care Europe GmbH, Milan). Results are expressed as antibody/β-tubulin ratio [179], [63].

2.10.5. Protein localization by immunofluorescence measurements

Immunofluorescence was performed as described by Ara et al. [180]. Firstly, Caco-2 cells were subjected to AAPH (25 mg/ml), CBD (100%) and CBDA (27%) preparations (5 and 10 μM) and combined AAPH/cannabinoid treatment, and TEER was monitored every 60 min as described in Section 2.10.2. After 5.5 hours, DMEM was removed from the filter, which was additionally washed 3 times with PBS (with calcium chloride and magnesium chloride). Samples were fixed adding methanol (3×500 μL on the apical and 3×500 μL of on the basolateral part) at -20°C. Solvent was removed and filters were dried at room temperature (~24°C) for 15 min, 0.1% BSA in PBS (with calcium chloride and magnesium chloride) was added and maintained for 10 min. Filters were carefully cut off and put in a humid chamber and 1% BSA in PBS (with calcium chloride and magnesium chloride) was added on the monolayer for 30 min to block non-specific sites. After that monolayer was washed 3×5 min with 0.1% BSA. 50 μL of the ZO-1 and occludin primary antibodies proteins solution at the final concentration of 20 μL/mL 1% BSA was added on the filters and incubated at room temperature (~24°C) for 60 min. After incubation, primary antibodies were removed and filters were washed 3×5 min with 0.1% BSA. 50 μL of secondary antibodies (FITC and TRITC) solution at the final concentration of 5 μL/mL 1% BSA was added on the filters and incubated in the dark place at room temperature (~24°C) for 60 min. After incubation, secondary antibodies were removed and filters were washed 3×5 min with 0.1% BSA and then with PBS (with calcium chloride and magnesium chloride). Microscope glass slides were prepared washing with dist.H_2O, ethanol and adding a drop of Prolong Gold antifade reagent. In dist.H_2O rapidly washed and dried filters were carefully put on the slide and the Prolong Gold antifade reagent was added to avoid bubbles and put the cover clips. Prior to microscoping, samples were stored in the fridge (4°C) with minimal exposure of light. Samples were analysed under a confocal microscope (LSM 700, Zeiss, Jena, Germany).

2.11. Experimental design

Response surface methodology (RSM) using central composite design (CCD) was employed to determine the effect of three independent variables on the SCE-CO_2 extract yield and to identify optimal SCE-CO_2 conditions for: (1) *C. sativa,* variety unspecified (mixture of blossoms, leaves and stems) and (2) *C. sativa 'Benico'* (mixture of seeds, blossoms and leaves). Extraction pressure (P), temperature (T) and time (t) were chosen as independent variables with three levels for each **Table 7**. The number of experiments is defined by the formula:

$N = (2^f + 2f + c)$, where: f is the number of factors; c is the number of center points

The complete design, consisting of 20 experimental runs with 8 factorial points, 6 axial points and 6 centre points, was established using the software Design-Expert trial version 8.0.7.1 (Stat–Ease Inc., Minneapolis, MN) as previously reported by Kraujalis and Venskutonis (2013) [181]. The data obtained from the CCD design was fitted with a second order polynomial equation, which can be expressed as:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i \neq 1\ j=1}^{4} \beta_{ij} x_i x_j$$

Where Y is the predicted response; β_0 is a constant; βi, βii, βij are the coefficients for linearity; xi and xj are independent variables.

Statistical significance of the model and model variables was determined at the 5% probability level ($p < 0.05$). The adequacy of the model was determined by evaluating the lack of fit coefficient and the Fisher test value (F-value) obtained from the analysis of variance. Extractions at every experimental point were performed in random order.

2.12. Statistical analysis

Extraction experiments, phytochemical composition analysis, CBD and CBDA preparation activity in Caco-2 cells measurements were performed in duplicate, cannabinoid and sugar analysis – in triplicate, antioxidant activity assessment experiments – at least in quadruplicate. Mean values and standard deviations were calculated using MS Excel 2003. One-way analysis of the variance (ANOVA), followed by the Tukey's posthoc test to compare the means that showed significant variation ($p < 0.05$), were performed using STATISTICA 8.0 software (2007).

III. RESULTS AND DISCUSSION

3.1. Isolation of cannabinoid and antioxidant fractions from various *C. sativa* cultivars

At the initial step of this research, a broad screening of 20 fiber-type hemp samples (**Table 6**), including *C. sativa* cultivars *'Finola', 'USO'* and *'Benico'* at different vegetation periods, mixture of *C. sativa* blossoms, leaves and stalks of unspecified cultivar and *C. sativa* processing by-products with different amounts of seed hulls was performed in order to select the most promising cultivar for further biorefining protocol development. The main evaluation criteria were: (1) yield of lipophilic fraction, obtained by means of SCE-CO_2; (2) *in vitro* antioxidant capacity and target cannabinoid (CBD and CBDA) content in SCE-CO_2 extracts and plant material before and after extraction (**Fig. 8**).

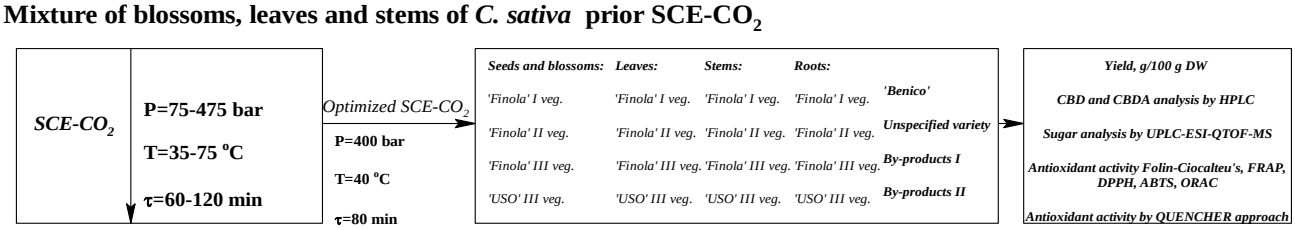


Fig. 8. Schematic representation of SCE-CO_2 extraction and analysis of various *C. sativa* samples

3.1.1. Optimisation of SCE-CO_2 parameters and model analysis

As discussed in Chapter 2.4, SCE-CO_2 is promising technique for oil, essential oil and targeted lipophilic constituent isolation from various plant material [13]. However, SCE-CO_2 efficiency depends on extraction temperature, pressure, time, particle size, etc., thus process parameter optimization is a critical step for the effective treatment. This was previously shown for hemp oil isolation from seeds and essential oil fractionation from blossoms of *C. sativa* cultivars 'Felina' and 'Fedora' [13], and also exemplified by our research group for amaranth seeds [181], black currant buds [182], and raspberry pomace [159]. For the SCE-CO_2 optimization, mixture of *C. sativa* blossoms, leaves and stalks of unspecified cultivar was used at this step of the research (**Fig. 8**). Central composite design (CCD) and response surface methodology (RSM) were employed to study the effect of three independent variables, namely pressure (P, 75-475 bar), temperature (T, 35-70 °C) and extraction time (τ, 60-120 min.), on the SCE-CO_2 extract yield and to determine optimal SCE-CO_2 parameters. The complete design consisted of 20 experimental runs with 8 factorial points, 6 axial points and 6 center points (**Table 8**). The ranges of variables were selected following the studies of Kraujalis [181], Porto and Decorti [13] and Porto and Voinovich [119].

Table 8. Hemp SCE-CO_2 extraction parameters by CCD and obtained SCE-CO_2 extract yields and CBD and CBDA amount in extracts

No.	SCE-CO_2 parameters			Yield	CBD		CBDA	
	Pressure, bar	*Temperature, °C*	*Time, min*	*g/100 g DW*	*mg/g extract*	*g/100 g DW*	*mg/g extract*	*g/100 g DW*
1	75	35	60	0.10 ± 0.00^{a}	$-^{nd}$	$-^{nd}$	$-^{nd}$	$-^{nd}$
2	275	70	90	20.80 ± 0.56^{f}	33.817 ± 1.893^{c}	0.703 ± 0.039^{c}	4.425 ± 0.250^{e}	0.092 ± 0.005^{cd}
3	275	52.5	90	18.11 ± 0.08^{c}	24.256 ± 2.601^{ab}	0.439 ± 0.047^{a}	3.097 ± 0.330^{cd}	0.056 ± 0.006^{b}
4	75	52.5	90	0.12 ± 0.00^{a}	$-^{nd}$	$-^{nd}$	$-^{nd}$	$-^{nd}$
5	275	52.5	60	15.28 ± 0.15^{b}	34.670 ± 0.963^{c}	0.530 ± 0.015^{ab}	2.307 ± 0.064^{bc}	0.035 ± 0.001^{a}
6	75	70	120	0.21 ± 0.00^{a}	$-^{n}$	$-^{n}$	$-^{n}$	$-^{n}$
7	275	52.5	90	18.18 ± 0.01^{c}	24.256 ± 2.601^{ab}	0.441 ± 0.047^{a}	3.097 ± 0.330^{cd}	0.056 ± 0.006^{b}
8	275	52.5	90	18.42 ± 0.00^{c}	24.265 ± 2.601^{ab}	0.447 ± 0.048^{a}	3.097 ± 0.330^{cd}	0.057 ± 0.006^{b}
9	75	70	60	0.18 ± 0.01^{a}	$-^{nd}$	$-^{nd}$	$-^{nd}$	$-^{nd}$
10	275	52.5	90	19.78 ± 0.06^{e}	24.256 ± 2.601^{ab}	0.480 ± 0.051^{ab}	3.097 ± 0.330^{cd}	0.061 ± 0.007^{b}
11	475	52.5	90	21.82 ± 0.18^{h}	21.965 ± 0.447^{ab}	0.479 ± 0.010^{ab}	4.895 ± 0.127^{e}	0.107 ± 0.003^{d}
12	75	35	120	0.11 ± 0.00^{a}	$-^{nd}$	$-^{nd}$	$-^{nd}$	$-^{nd}$
13	475	70	60	21.64 ± 0.57^{gh}	23.341 ± 0.582^{ab}	0.505 ± 0.013^{ab}	6.181 ± 0.144^{f}	0.134 ± 0.003^{e}
14	275	52.5	120	19.17 ± 0.02^{d}	20.384 ± 2.117^{a}	0.391 ± 0.041^{a}	2.940 ± 0.285^{c}	0.056 ± 0.005^{b}
15	275	35	90	18.57 ± 0.31^{c}	23.991 ± 0.025^{ab}	0.446 ± 0.000^{a}	1.302 ± 0.025^{a}	0.024 ± 0.000^{a}
16	475	70	120	21.58 ± 0.05^{gh}	20.662 ± 1.320^{a}	0.446 ± 0.028^{a}	4.768 ± 0.270^{e}	0.103 ± 0.006^{cd}
17	275	52.5	90	19.33 ± 0.02^{de}	24.256 ± 2.601^{ab}	0.469 ± 0.050^{a}	3.097 ± 0.330^{cd}	0.060 ± 0.006^{b}
18	475	35	60	20.42 ± 0.13^{f}	25.227 ± 0.951^{ab}	0.515 ± 0.019^{ab}	1.592 ± 0.084^{ab}	0.033 ± 0.002^{a}
19	275	52.5	90	19.30 ± 0.01^{de}	24.256 ± 2.601^{ab}	0.468 ± 0.050^{a}	3.097 ± 0.330^{cd}	0.060 ± 0.006^{b}
20	475	35	120	20.48 ± 0.36^{f}	25.546 ± 0.137^{ab}	0.523 ± 0.003^{ab}	1.716 ± 0.024^{ab}	0.035 ± 0.000^{a}
Optimal conditions:								
	400	**40**	**80**	**21.61 ± 0.03**	**28.514 ± 1.550^{bc}**	**0.616 ± 0.03^{bc}**	**3.967 ± 0.142^{de}**	**0.086 ± 0.003^{c}**

$-^{nd}$: not detected; Different superscript letters within the same line indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$)

Experimental data in **Table 8** show that 0.1-21.82 g/100 g DW of lipophilic fraction could be obtained from the unspecified *C. sativa* cultivar (mixture of blossoms leaves and stalks under different SCE-CO_2 conditions, containing 20.662-33.817 mg/g extract (0.391-0.703 g/100 g DW) of CBD and 1.302-6.181 mg/g extract (0.024-0.134 g/100 g DW) of CBDA. The adequacy of the model was evaluated by the total determination coefficient (R^2) value of 0.9932, indicating a reasonable fit of the model to the experimental data. Adjusted coefficient of determination (R^2, a measure of the amount of variation about the mean) of 0.9871 is in agreement with the predicted coefficient of determination (R^2) of 0.9584. Model evaluation is presented in the analysis of variance (**Table 9**).

Table 9. Analysis of variance table for response surface quadratic model

Source	Sum of Squares	df	Mean Square	F-Value	*p*-value
Model	1440.31	9	160.03	162.00	< 0.0001*
Pressure (P, bar)	1107.12	1	1107.12	1120.75	< 0.0001*
Temperature (T, °C)	2.24	1	2.24	2.26	0.1633**
Time (τ, min)	1.54	1	1.54	1.56	0.2396**
PT	0.57	1	0.57	0.58	0.4641**
Aτ	$2.00 \cdot 10^{-4}$	1	$2.00 \cdot 10^{-4}$	$2.025 \cdot 10^{-4}$	0.9889**
Tτ	$1.250 \cdot 10^{-3}$	1	$1.250 \cdot 10^{-3}$	$1.265 \cdot 10^{-3}$	0.9723**
P^2	164.40	1	164.40	166.42	< 0.0001*
T^2	2.66	1	2.66	2.69	0.1320**
τ^2	6.00	1	6.00	6.07	0.0334*
Residual	9.88	10	0.99		
Lack of fit	7.40	5	1.48	2.98	0.1276**
Pure error	2.48	5	0.50		
Total SS	1450.19	19			

*: significant; **: not significant

The significance of each model was determined using the Student test (p-value). The analysis of the quadratic regression models for oil yield showed that the model was significant ($p<0.05$) with an F-value of 2.98 and the '*lack of fit*' was not significant relative to the pure error, with a p-value of 0.1276. The model shows that the factor with the largest effect on oil yield was extraction pressure ($p<0.05$, F=1120.75), while extraction temperature (T, $p<0.1633$) and time (τ, $p<0.2396$) are not significant variables. Interactions between pressure and temperature (PT) and pressure and time (Pτ) and temperature and time (Tτ) are not significant ($p>0.05$). The second-order terms pressure (P^2) and time (τ^2) were significant ($p<0.05$). Extraction pressure has the highest positive effect for yield, while the effect of temperature and time is less important. These results are in agreement with studies of Kraujalis [181] and Porto and Vainovich [119], reporting that extraction time and pressure are the most important parameters in SCE-CO_2 extraction [183]. Predicted values of fiber type hemp SCE-CO_2 extract yield, calculated using a second order polynomial equation and regression model, were compared with experimental values (**Fig. 9**).

Second order polynomial regression model – an empirical relationship between dependent and the independent test variables (P, T, τ) – is given in the following equation:

$$Yield, \% = 18.79 + 10.52 \times P \times 0.47 \times T \times 0.39 \times t \times 0.27 \times P \times T - 0.005 \times P \times \tau - 0.012 \times T \times \tau - 7.73 \times P^2 + 0.98 \times T^2 - 1.48 \times \tau^2$$

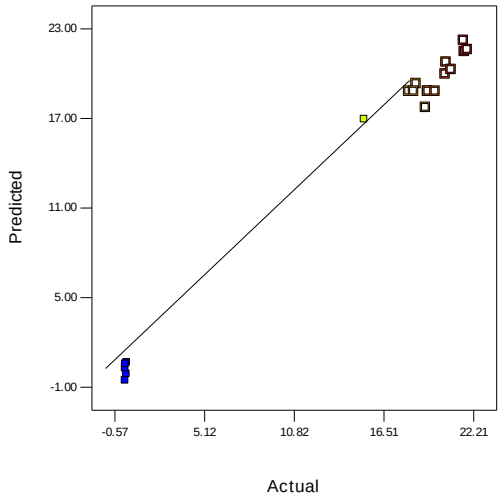


Fig. 9. Comparison between predicted and observed *C. sativa* SCE-CO_2 extract yield (g/100 g DW)

Response surface plots, obtained by RSM and reporting the effect of extraction pressure, temperature, and time on SCE-CO_2 extract yield, are depicted in **Fig. 10**.The graphs were obtained by fixing two variables at coded zero level, while varying the remaining one variable and predicting the response variable. **Fig. 10** (A) illustrates the effect of extraction temperature and pressure on extract yield at constant 90 min extraction time. In this case, extraction pressure has significant influence on extract amount, while extraction temperature has no significant impact on extract yield. Performing extraction at lower pressures, the increase of temperature has slightly negative effect on the yield, while at higher pressures the temperature has some positive effect. Too high pressure has a slightly negative effect, due to the highly compressed CO_2 facilitates solute–solvent repulsion [119, 124, 183, 184]. **Fig. 10** (B) demonstrates that the extract content increases by increasing pressure at constant temperature of 52.5 °C, while time has slightly positive effect on extract yield. It was indicated that continuation of extraction after 90 min has no important effect on the extract yield [182]. **Fig. 10** (C) demonstrates that the yield increases by increasing time up to 90 min at constant pressure of 275 bar. After 90 min time has negligible effect on the extract yield, while increasing of temperature has positive effect on the extract amount.

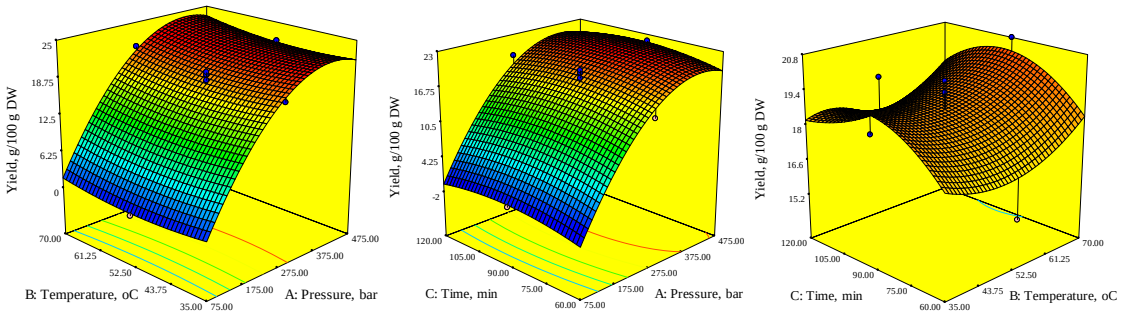


Fig. 10. 3D RSM plots of the dependencies of hemp extract yield on temperature/pressure (A), time/pressure (B) and time/temperature (C)

Considering all responses, optimal conditions to obtain the highest SCE-CO_2 extract yield are extraction pressure 400 bar, temperature 40°C and time 80 min. Under these conditions, 21.61 g/100 DW of oil can be isolated from mixture of blossoms, leaves and stalks of unspecified *C. sativa* cultivar, containing 28.514 mg/g (0.616 g/100 g DW) and 3.967 mg/g (0.086 g/100 g DW) of CBD and CBDA, respectively. Optimal SCE-CO_2 temperature and time are in agreement with studies of Da Porto and Voinovich, who reported that SCE-CO_2 under optimized pressure of 300 bar, temperature of 40°C and extraction time of 60 min amounts 21.05 g of lipophilic fraction from 100 g DW of hemp '*Felina'* seeds (0.71 mm particle size, *III* vegetation period) [119]. Similar results were obtained by Da Porto and Vainovich, where SCE-CO_2 parameters for hemp seed oil isolation from *'Felina'* cultivar were optimized at the following levels: pressure 250-350 bar, temperature 40-60°C, particle size 0.59-0.83 mm. Under optimal conditions (300 bar, 40°C and 0.71 mm and 60 min) oil yield was equal to 21.05 % [119] [118]. [155]. Authors reported that particle size and pressure had highest influence for extracted oil content. In addition it was observed that too small particles (<0.59 mm) and too high pressure (>300 bar) has negative effect for extractable oil content. Studies reported by Porto and Decorti (2009) demonstrate 16.48 g/100 g DW yield, isolated under the following SCE-CO_2 conditions: 300 bar, 80 °C, 48 min. Therefore, variation in yields could be partially explained by the differences in sample preparation and handling procedures applied, as well hemp variety and anatomical part of plant utilized [185].

3.1.2. SCE-CO_2 extract and cannabinoid yields under optimized conditions

Yields and cannabinoid (CBD and CBDA) content in SCE-CO_2 extracts and plant material before and after extraction in various *C. sativa* samples under the optimized SCE-CO_2 conditions are reported in **Table 10**.

Table 10. SCE-CO_2 extract yields and CBD and CBDA amount in different anatomical parts of *C. sativa 'Finola', 'USO', 'Benico'* cultivars and by-product extracts and plant material before and after SCE-CO_2

Samples	Yield	CBD			CBDA			
		SCE-CO_2 extracts	Plant material		SCE-CO_2 extracts		Plant material	
			Before SCE-CO_2	After SCE-CO_2			Before SCE-CO_2	After SCE-CO_2
	g/100 g DW	*mg/g extract*	*g/100 g DW*	*g/100 g DW*	*mg/g extract*	*g/100 g DW*	*g/100 g DW*	*g/100 g DW*
Seeds and blossoms:								
'Finola' I veg.	2.11 ± 0.04^{c}	66.601 ± 0.896^{j}	0.140 ± 0.002^{f}	0.117 ± 0.004^{f}	0.003 ± 0.000^{b}	133.770 ± 3.736^{h}	0.282 ± 0.008^{ef}	0.328 ± 0.010^{e}	0.117 ± 0.005^{e}
'Finola' II veg.	2.94 ± 0.08^{d}	39.251 ± 1.546^{i}	0.115 ± 0.005^{de}	0.113 ± 0.001^{f}	0.003 ± 0.000^{b}	302.688 ± 10.933^{j}	0.890 ± 0.032^{i}	0.955 ± 0.009^{h}	0.205 ± 0.007^{g}
'Finola' III veg.	7.70 ± 0.08^{g}	27.379 ± 1.387^{g}	0.211 ± 0.011^{g}	0.167 ± 0.002^{h}	0.002 ± 0.000^{ab}	101.856 ± 5.159^{fg}	0.784 ± 0.040^{h}	0.963 ± 0.012^{h}	0.203 ± 0.004^{g}
'USO' III veg.	14.83 ± 0.23^{b}	3.400 ± 0.034^{b}	0.050 ± 0.001^{c}	0.042 ± 0.002^{c}	0.001 ± 0.000^{a}	9.446 ± 0.161^{ab}	0.140 ± 0.002^{d}	0.161 ± 0.006^{c}	0.050 ± 0.002^{c}
Leaves:									
'Finola' I veg.	1.81 ± 0.05^{c}	35.039 ± 2.383^{h}	0.064 ± 0.004^{c}	0.045 ± 0.001^{c}	0.001 ± 0.000^{a}	57.945 ± 4.045^{d}	0.105 ± 0.007^{c}	0.191 ± 0.003^{cd}	0.066 ± 0.006^{d}
'Finola' II veg.	2.50 ± 0.07^{c}	21.329 ± 1.305^{f}	0.053 ± 0.003^{c}	0.059 ± 0.000^{d}	0.002 ± 0.000^{ab}	108.784 ± 2.324^{g}	0.272 ± 0.006^{e}	0.374 ± 0.004^{f}	0.114 ± 0.001^{e}
'Finola' III veg.	3.54 ± 0.08^{e}	27.877 ± 1.057^{g}	0.099 ± 0.004^{d}	0.078 ± 0.003^{e}	0.002 ± 0.000^{ab}	98.810 ± 6.923^{f}	0.350 ± 0.025^{g}	0.538 ± 0.017^{g}	0.125 ± 0.001^{e}
'USO' III veg.	3.96 ± 0.12^{e}	3.733 ± 0.015^{bc}	0.015 ± 0.000^{ab}	0.009 ± 0.000^{a}	-nd	78.632 ± 0.871^{e}	0.311 ± 0.003^{f}	0.388 ± 0.003^{f}	0.001 ± 0.000^{a}
Stalks:									
'Finola' I veg.	0.53 ± 0.03^{ab}	2.240 ± 0.000^{a}	0.001 ± 0.000^{a}	-nd	-nd	4.076 ± 0.000^{ab}	0.002 ± 0.000^{a}	0.002 ± 0.000^{a}	0.001 ± 0.000^{a}
'Finola' II veg.	0.62 ± 0.03^{b}	0.916 ± 0.000^{a}	0.001 ± 0.000^{a}	-nd	0.001 ± 0.000^{a}	4.155 ± 0.000^{ab}	0.003 ± 0.000^{a}	0.002 ± 0.001^{a}	0.003 ± 0.000^{a}
'Finola' III veg.	0.49 ± 0.02^{ab}	7.015 ± 0.005^{dc}	0.003 ± 0.000^{a}	0.001 ± 0.000^{a}	0.001 ± 0.000^{a}	21.607 ± 0.014^{c}	0.011 ± 0.001^{a}	0.007 ± 0.002^{a}	0.003 ± 0.000^{a}
'USO' III veg.	0.69 ± 0.01^{b}	3.298 ± 0.000^{b}	0.002 ± 0.000^{a}	0.002 ± 0.000^{a}	-nd	6.303 ± 0.003^{ab}	0.004 ± 0.000^{a}	0.005 ± 0.001^{a}	-nd
Roots:									
'Finola' I veg.	0.51 ± 0.01^{ab}	1.053 ± 0.000^{ab}	0.001 ± 0.000^{a}	-nd	$< 0.001^{a}$	1.275 ± 0.000^{a}	0.001 ± 0.000^{a}	-nd	-nd
'Finola' II veg.	0.62 ± 0.00^{b}	1.402 ± 0.000^{ab}	0.001 ± 0.000^{a}	-nd	0.001 ± 0.000^{a}	1.590 ± 0.000^{ab}	0.001 ± 0.000^{a}	-nd	-nd
'Finola' III veg.	0.34 ± 0.02^{ab}	1.374 ± 0.000^{ab}	$< 0.001^{a}$	-nd	-nd	1.275 ± 0.000^{a}	$< 0.001^{a}$	-nd	-nd
'USO' III veg.	0.18 ± 0.01^{a}	1.371 ± 0.000^{ab}	$< 0.001^{a}$	-nd	$< 0.001^{a}$	1.780 ± 0.000^{ab}	$< 0.001^{a}$	-nd	-nd
'Benico'*	7.51 ± 0.06^{g}	17.422 ± 0.423^{e}	0.131 ± 0.003^{ef}	0.140 ± 0.005^{g}	0.002 ± 0.001^{ab}	256.452 ± 0.081^{i}	1.923 ± 0.001^{j}	2.35 ± 0.041^{i}	0.287 ± 0.016^{h}
Unspecified variety	21.61 ± 0.03^{i}	28.514 ± 1.550^{g}	0.616 ± 0.03^{h}	0.528 ± 0.014^{i}	0.019 ± 0.001^{d}	3.967 ± 0.142^{ab}	0.086 ± 0.003^{bc}	0.195 ± 0.005^{d}	0.175 ± 0.007^{f}
By-products I*	5.47 ± 0.07^{f}	5.783 ± 0.277^{c}	0.032 ± 0.002^{b}	0.029 ± 0.000^{b}	0.011 ± 0.001^{c}	10.367 ± 0.416^{b}	0.057 ± 0.002^{b}	0.099 ± 0.007^{b}	0.019 ± 0.001^{b}
By-products II**	21.93 ± 0.65^{i}	1.382 ± 0.055^{ab}	0.030 ± 0.001^{b}	0.027 ± 0.001^{b}	0.003 ± 0.000^{b}	5.480 ± 0.173^{ab}	0.120 ± 0.004^{cd}	0.160 ± 0.013^{c}	0.040 ± 0.003^{c}

-nd: not detected; *: mixture of seeds, blossoms and leaves; **: C. sativa mixture of blossoms, leaves and Stalks; ***: C. sativa seed hulls (major part); ****: C. sativa seed hulls (minor part). Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$).

On the basis of **Table 10** the obtained results, lipophilic fraction yield in various *C. sativa* samples tested ranged from 0.18 to 21.93 g/100 g DW. Hemp processing by-products (21.93 g/100 g DW) and *C. sativa* material (21.61 g/100 g DW), used to optimize SCE-CO_2 parameters, showed the highest extract yield, followed by *'Benico'* cultivar (7.51 g/100 g DW), and seeds and blossoms (on average 6.89 g/100 g DW), leaves (on average 2.9 g/100 g DW), stalks (on average 0.58 g/100 g DW) and roots (on average 0.41 g/100 g DW) from *'Finola'* and *'USO'* cultivars. Generally, it has been observed that the later vegetation period has positive effect for extract yield: *I veg* (on average 1.24 g/ 100 g DW) < *II veg* (1.67 g/100 g DW) < *III veg*.(3.2 g/100 g DW). These results were expected and could be explained by maturation of plant, because it is well known that after harvesting hemp accumulate highest content of oil [26]. Also, seeds and blossoms (on average 6.89 g/100 g DW) are characterized by higher extract yield than leaves (on average 2.9 g/100 g DW), which could be explained by higher concentration of lipophilic constituents in the later anatomical parts of plant. The least content of extract was isolated from stalks and roots (on average, 0.58 and 0.41 g/100 g DW), respectively.

Looking at the content of target cannabinoids under optimal extraction conditions, CBD amounted from 0.916 to 66.601 mg, CBDA – from 1.275 to 302.688 mg per gram of SCE-CO_2 extract (**Table 10**.). When recalculated per 100 g of ground plant prior SCE-CO_2, these quantities were equal to 0.001-0.211 and 0.001-1.923 g/100 g DW of CBD and CBDA respectively. Different cultivar of *C. sativa* is important for CBD and CBDA content in plant material: *'Benico'* (0.14 and 2.35 g/100 g DW, CBD and CBDA respectively) > *'Finola'* (0.09 and 0.558 g/100 g DW, CBD and CBDA respectively) > *'USO'* (0.0255 and 0.125 g/100 g DW, CBD and CBDA respectively). Comparing different anatomical parts of fiber type hemps, generally the highest content of CBD was indicated in seeds and blossoms (0.042-0.167 g/100 g DW), which is up to 2.6 times higher as compared to leaves (0.009-0.078 g/100 g DW). Very low concentrations of CBD (0.001-0.002 g/100 g DW) were identified in stalks and not detected in roots. CBDA distribution in different anatomical parts was similar to CBD (**Table 10**): seeds and blossoms (0.161-0.963 g/100 g DW) > leaves (0.191-0.538 g/100 g DW) > stalks (0.002-0.007 g/100 g DW) > roots (not identified). It was noticed that CBD and CBDA content in different fiber type hemp anatomical parts highly depends on the vegetation period and cultivar tested. For example, in *'Finola'* seeds and blossoms of *I* and *II* vegetation CBD content is 0.140 and 0.115 g/100 g D, respectively, and increase during *III* vegetation up to 0.211 g/100 g DW, while CBDA content increase up to 3.15 fold during *II* vegetation (0.890 g/100 g DW) with no particular changes observed for *III* vegetation samples (0.784 g/100 g DW). These results could be explained by partial CBDA conversion to CBD, because as reported in Chapter 1.3 CBDA is a precursor for CBD [6]. In *'Finola'* leaves, CBD and

CBDA amount increase with every vegetation period: *I veg.* (0.064 and 0.191 g/100 g DW, of CBD and CBDA respectively) < *II veg*. (0.053 and 0.374 g/100 g DW, of CBD and CBDA respectively) < *III veg.* (0.099 and 0.538 g/100 g DW of CBD and CBDA respectively), although CBD content in *II veg*. is lower as compared to *I veg.*, however the difference between I and II veg. is not significant. CBD and CBDA content in hemp stalks is very low and vegetation period has no impact, while in roots those compounds were not identified. Looking at the effect of cultivar tested, CBD and CBDA concentration in *III veg*. of *'Finola'* seeds and blossoms is by 76.3 and 85.6%, respectively higher as compared to *'USO'* corresponding samples. CBD and CBDA concentration in *III veg*. of *'Finola'* leaves is by 84.8 and 27.8%, respectively higher as compared to *'USO'* corresponding samples. No significant differences were determined comparing CBD and CBDA content in stalks and roots of *'Finola'* and *'USO'* cultivars. CBD content in *'Benico'* sample is by 15% lower as compared to average content of *III veg. 'Finola'* seeds, blossoms and leaves, and by 81% higher as compared to average content of *III veg. 'USO'* seeds and blossoms and leaves. However, this cultivar distinguishes by the highest CBDA content among all samples tested: by 71-88% higher as compared to average content of *III veg. 'Finola'* and *USO'* samples. As reported by Gruzdevienė and Jankauskienė [18], CBD amount in seeds and blossoms sample in *'Benico'* and *'USO'* cultivars was 1.8 and 0.24%, respectively. The results of this study show different ratios, which could be partly explained by different growing, sampling and storage conditions of hemp material prior to the analysis.

With an exception of mixture of blossoms, leaves and stalks (unspecified *C. sativa* cultivar) used for SCE-CO_2 process optimization, all tested samples demonstrated higher CBDA content as compared to CBD: 2-9 fold for *'Finola'* seeds and blossoms (on average, 0.749 g CBDA /100 g DW of plant material before SCE-CO_2) and leaves (0.551 g/100 g DW of plant material before SCE-CO_2), 4-43 fold for *'USO'* seeds and blossoms (0.161 g/100 g DW of plant material before SCE-CO_2) and leaves (0.388 g/100 g DW), 3-6 fold for hemp by–products (0.129 g/100 g DW of plant material before SCE-CO_2). Vice versa, unspecified *C. sativa* cultivar accumulated on average 5-fold lower amount of CBDA, as compared to other fiber type hemp samples of different cultivars, anatomical parts and vegetation periods tested. Furthermore, the content of parent compound CBDA in the mixture used for SCE-CO_2 optimization was 3-fold lower as compared to its decarboxylated form CBD. These results could be explained by decarboxylation of CBDA to CBD (**Fig. 11**) during storage at the higher relative humidity of the air, different growing, storage and sampling conditions [186].

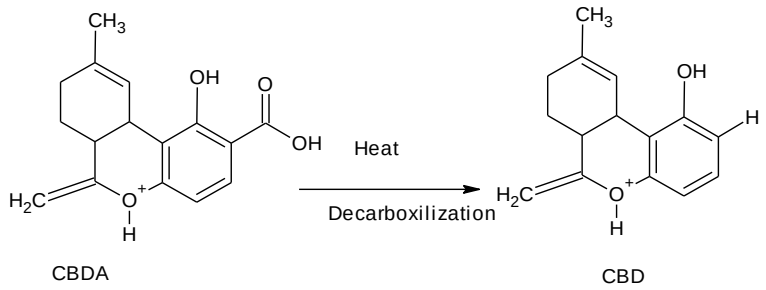


Fig. 11. CBDA decarboxylation to CBD

Looking at the cannabinoid content in plant material before extraction, CBD varied from 0.001 to 0.528 g/100 g DW and CBDA – from 0.002 to 2.5 g/100 g DW. The quantities of cannabinoids in the residues after extraction were rather low, amounting only 0.001-0.0019 g of CBD and 0.001-0.287 g of CBDA per 100 g of sample. The share of SCE-CO_2 extract to the overall target cannabinoid content varied from 0.001 to 0.616 g/100 g DW and from 0.001 to 1.923 g/100 g DW, for CBD and CBDA, respectively. Thus, nearly all CBD (~98%) and the major part of CBDA (60-80%) was isolated from raw material by SCE-CO_2 and concentrated in lipophilic fraction. This suggests that SCE-CO_2 is a suitable technique for target cannabinoid isolation and concentration in lipophilic fraction from various *C. sativa* cultivars. In addition, on the basis of this data is observed that the high content of major fiber-type cannabinoids CBD and particularly CBDA was indicated for *'Benico'* cultivar, showing its potential for further biorefining protocol development.

3.1.3. In vitro antioxidant capacity of SCE-CO_2 extracts and plant material

For the purposes of this research, *in vitro* antioxidant properties of various SCE-CO_2 extracts, isolated under optimized extraction conditions (400 bar. 40°C, 80 min), were assessed as radical scavenging capacity, reducing power and total phenolic content, employing respectively $DPPH^{\bullet}$, $ABTS^{\bullet+}$ ORAC, FRAP and Folin-Ciocalteu's assays. As discussed in Chapter 2.5, solid residue after extraction may retain a considerable part of antioxidatively active constituents, thus the potential of extracts was additionally compared with the *in vitro* antioxidant capacity of plant material before and after extraction. For these purposes, so-called QUENCHER approach was employed for the total phenolic content, ferric reducing power (FRAP), $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and ORAC radical scavenging capacity measurements [167]. The results of these assays are presented in **Table 11**, **Table 12** and **Table 13**, respectively.

Folin–Ciocalteu's is relatively simple electron transfer-based assay, commonly applied to assess the TPC of natural products [168]. The reaction between phenolic compound and Folin-Ciocalteu's reagent (redox reagent) leads to the formation of blue complex, which colour intensity

can be measured visible-light spectrophotometry at 628-780 nm wavelengths [187, 188, 189]. The measured absorbance is directly proportional to the amount of phenolic constituents in the sample. Previously Folin–Ciocalteu's method was suggested for standardization and characterization of plants according to the content of polyphenols [190, 191, 192]. However, various compounds, e.g. reducing sugars, are known to reduce the Folin– Ciocalteu's reagent as well, therefore this assay in general measures reducing capacity of the whole sample [154]. As presented in **Table 11**, the TPC ranged from 19.05 to 124.89 mg GAE per gram of SCE-CO_2 extract, with mean of 72.01 and 61.29 mg GAE/g extract across the samples of seeds and blossoms and leaves of different vegetation, respectively. Taking into account the yield of extract (**Table 11**), this amount is in the range 1.11-6.97 mg GAE/g DW plant material in the following order: leaves (1.11-2.38 mg GAE/g DW) from cultivars *'Finola'* and *'USO'* < seeds and blossoms (1.89-4.18 mg GAE/g DW) from cultivars *'Finola'* and *'USO'* < by-products (average 4.55 mg GAE/g DW) < unspecified *C. sativa* cultivar used for SCE-CO_2 optimization (5.97 mg GAE/g DW) < *'Benico'* cultivar (6.97 mg GAE/g DW). These results are in agreement with studies reported by Srivastava and Chauhan (2012), who showed that total phenolic content of *'Benico'* leaves and blossoms are 9.62 and 13.5 mg/g extract, respectively. In addition, these authors also reported higher total phenolic content activity for *'Benico'* seeds and blossoms as compared to leaves of *'Finola'* cultivar [98]. Data in **Table 11** show that the activities of starting material before SCE-CO_2 were equal to 19.32-33.21 mg GAE/g DW and 24.26 -35.88 mg GAE/g DW in seeds and blossoms and leaves respectively, 6.71-5.39 mg GAE/g DW and 5.16-3.76 mg GAE/g DW in stalks and roots respectively, 8.67-9.76 mg GAE/g DW in by-products, 35.49 mg GAE/g DW in *'Benico'* sample and 8.08 mg GAE/g DW in sample of unspecified *C. sativa* cultivar and followed this decreasing order: *'Benico'* cultivar > leaves from cultivars *'Finola'* and *'USO'* > seeds and blossoms from cultivars *'Finola'* and *'USO'* > by-products > unspecified *C. sativa* variety (used for SCE-CO_2 optimization) > roots > stalks. The share of SCE-CO_2 extract to the total phenolic content varied from 1.11 to 6.97 mg GAE/g DW. The SCE-CO_2 reduced the TPC values of hemp by 12.5% in *'Benico'* sample (32.59 mg GAE/g DW, equal to 30.14 mg GAE/g DW of plant material prior SCE-CO_2), 3.3-13.3% in seeds and blossoms (19.68-36.27 mg GAE/g DW or 16.76-35.20 mg GAE/g DW prior SCE-CO_2), by 0.1-10.23% in leaves (24.73-33.39 mg GAE/g DW or 23.85-32.07 mg GAE/ g DW prior SCE-CO_2), by 0.39-10.14 %, 2.5-46.23 %, 30.7-36.7% and 9.0% for stalks, by-products and sample of unspecified *C. sativa* variety, respectively. Results in this study are in agreement with results reported by Mihoc and Pop (2013), who showed that hemp seed oil, extracted with petroleum ether, exerted TPC value of 3.61 mg GAE/g oil, which is relatively similar to results obtained in our study [193].

Table 11. Total phenolic content (TPC) and ferric reducing antioxidant power (FRAP) of various hemp samples before and after SCE-CO_2, and SCE-CO_2 extracts, expressed as gallic acid equivalents (TPC, mg GAE/g) and Trolox equivalent antioxidant capacity (TEAC, mg TE/g), respectively

Samples	TPC				$TEAC_{FRAP}$			
	SCE-CO_2 extracts		Plant material		SCE-CO_2 extracts		Plant material	
			Before SCE-CO_2	*After SCE-CO_2*			*Before SCE-CO_2*	*After SCE-CO_2*
	mg GAE/g extract	*mg GAE/g DW*	*mg GAE/g DW*	*mg GAE/g DW**	*mg TE/g extract*	*mg TE/g*	*mg TE/g DW*	*mg TE/g DW**
***Seeds and blossoms**:*								
'Finola' I veg.	89.81 ± 2.24^{f}	1.89 ± 0.05^{c}	28.46 ± 1.53^{i}	27.55 ± 1.94^{e}	63.98 ± 0.57^{d}	1.35 ± 0.01^{a}	71.98 ± 0.83^{i}	56.83 ± 0.88^{h}
'Finola' II veg.	124.89 ± 2.02^{g}	3.67 ± 0.06^{g}	33.21 ± 2.16^{j}	36.27 ± 2.80^{g}	92.96 ± 1.37^{e}	2.73 ± 0.04^{b}	76.11 ± 0.42^{j}	58.53 ± 2.20^{h}
'Finola' III veg.	54.31 ± 1.77^{d}	4.18 ± 0.14^{h}	22.75 ± 1.11^{h}	20.08 ± 1.81^{d}	66.03 ± 3.88^{d}	5.08 ± 0.30^{e}	56.73 ± 1.48^{g}	32.60 ± 0.50^{e}
'USO' III veg.	19.05 ± 0.44^{a}	2.82 ± 0.07^{f}	19.32 ± 1.21^{g}	19.68 ± 1.04^{d}	7.71 ± 1.59^{a}	1.14 ± 0.24^{a}	42.11 ± 0.91^{f}	40.84 ± 4.02^{f}
Leaves:								
'Finola' I veg.	61.62 ± 2.42^{e}	1.11 ± 0.04^{a}	27.42 ± 0.46^{i}	27.62 ± 2.43^{e}	50.18 ± 0.86^{c}	0.90 ± 0.02^{a}	66.47 ± 1.30^{h}	63.60 ± 1.32^{i}
'Finola' II veg.	60.51 ± 1.15^{e}	1.51 ± 0.03^{b}	31.16 ± 0.18^{j}	28.26 ± 0.27^{e}	115.71 ± 1.17^{f}	2.89 ± 0.03^{b}	66.17 ± 2.67^{h}	51.06 ± 0.47^{g}
'Finola' III veg.	62.03 ± 0.37^{e}	2.17 ± 0.01^{d}	24.26 ± 0.18^{h}	24.73 ± 1.14^{e}	115.10 ± 1.42^{f}	4.03 ± 0.05^{c}	56.83 ± 0.48^{g}	53.60 ± 1.47^{gh}
'USO' III veg.	60.98 ± 0.73^{e}	2.38 ± 0.03^{e}	35.88 ± 1.57^{k}	33.39 ± 1.20^{f}	114.04 ± 0.73^{f}	4.45 ± 0.03^{c}	80.59 ± 1.37^{k}	73.24 ± 1.49^{j}
Stalks:								
'Finola' I veg.	$-^{na}$	$-^{na}$	6.27 ± 0.36^{bcd}	6.49 ± 0.12^{c}	$-^{na}$	$-^{na}$	15.17 ± 0.32^{cd}	13.18 ± 1.05^{bc}
'Finola' II veg.	$-^{na}$	$-^{na}$	6.71 ± 0.28^{cde}	6.09 ± 0.24^{bc}	$-^{na}$	$-^{na}$	16.58 ± 1.39^{d}	13.57 ± 0.50^{bc}
'Finola' III veg.	$-^{na}$	$-^{na}$	5.39 ± 0.26^{ab}	5.37 ± 0.23^{abc}	$-^{na}$	$-^{na}$	13.40 ± 0.16^{bc}	13.14 ± 0.32^{bc}
'USO' III veg.	$-^{na}$	$-^{na}$	6.37 ± 0.42^{bcde}	6.48 ± 0.33^{c}	$-^{na}$	$-^{na}$	16.79 ± 0.10^{d}	15.62 ± 1.38^{c}
Roots:								
'Finola' I veg.	$-^{na}$	$-^{na}$	4.86 ± 0.33^{abc}	4.75 ± 0.17^{abc}	$-^{na}$	$-^{na}$	13.16 ± 0.52^{bc}	13.40 ± 0.11^{bc}
'Finola' II veg.	$-^{na}$	$-^{na}$	5.16 ± 0.13^{abc}	3.60 ± 0.02^{ab}	$-^{na}$	$-^{na}$	11.94 ± 0.15^{b}	11.00 ± 0.08^{b}
'Finola' III veg.	$-^{na}$	$-^{na}$	4.08 ± 0.27^{ab}	2.92 ± 0.21^{a}	$-^{na}$	$-^{na}$	12.02 ± 0.08^{b}	10.33 ± 0.08^{ab}
'USO' III veg.	$-^{na}$	$-^{na}$	3.76 ± 0.23^{a}	2.57 ± 0.12^{a}	$-^{na}$	$-^{na}$	8.77 ± 0.28^{a}	6.57 ± 0.19^{a}
'Benico'**	92.91 ± 1.53^{f}	6.97 ± 0.11^{j}	35.49 ± 1.37^{jk}	32.59 ± 0.18^{f}	43.73 ± 2.41^{c}	5.93 ± 0.07^{d}	80.12 ± 0.94^{k}	76.42 ± 3.31^{j}
Unspecified variety ***	27.64 ± 0.27^{b}	5.97 ± 0.06^{i}	8.08 ± 0.39^{d}	7.34 ± 0.35^{c}	63.98 ± 0.57^{d}	3.99 ± 0.20^{c}	14.88 ± 0.16^{b}	56.83 ± 0.88^{h}
By-products I****	30.22 ± 0.62^{b}	1.65 ± 0.03^{b}	8.67 ± 0.41^{edf}	6.33 ± 0.47^{bc}	92.96 ± 1.37^{e}	1.01 ± 0.45^{a}	21.89 ± 0.92^{d}	58.53 ± 2.20^{h}
By-products II*****	33.96 ± 0.83^{c}	7.44 ± 0.18^{k}	9.76 ± 0.60^{f}	7.47 ± 0.21^{c}	66.03 ± 3.88^{d}	2.05 ± 0.52^{b}	17.18 ± 0.31^{d}	32.60 ± 0.50^{e}

$-^{na}$: not available; *: mg GAE/g or mg TE/g sample after SCE-CO_2; **: mixture of seeds, blossoms and leaves; ***: C. sativa mixture of blossoms, leaves and stalks; ****: C. sativa seed hulls (major part); *****- C. sativa seed hulls (minor part). Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$).

Ferric reducing antioxidant power (FRAP) measures total reducing power of plant extracts [171] [194], visually observed as formation of blue coloured product via reduction of ferric 2,4,6-tripyridyl-s-triazine (TPTZ). Increasing reducing power is related with the degree of hydroxylation and extent of conjugation in polyphenols [194]. FRAP cannot detect compounds that act as H transfer, radical quenching [192, 194]. As presented in **Table 11**, the $TEAC_{FRAP}$ values ranged from 7.71 to 115.10 mg TE per gram of SCE-CO_2 extract, with mean of 74.32 and 98.75 mg TE/g extract across different vegetation seeds and blossoms, and leaves samples, respectively. When recalculated per g of plant material, these values amounted from 0.90 to 5.93 mg TE. The trend of $TEAC_{FRAP}$ in extracts of different anatomical parts is as follows: *'Benico'* (5.93 mg TE/g DW) > leaves (mean of 3.06 mg TE/g DW across *'Finola'* and *'USO'* cultivars) > seeds and blossoms (mean of 2.5 06 mg TE/g DW across *'Finola'* and *'USO'* cultivars) > unspecified *C. sativa* cultivar for SCE-CO_2 optimization (3.99 mg TE/g DW) > by-products (mean of 3.06 mg TE/g DW). In agreement to these observations, $TEAC_{FRAP}$ of starting material before SCE-CO_2 was in the range of 80.12 mg TE/g DW of *'Benico'* sample, 56.83-80.59 mg TE/g DW of leaves *'Finola'* and *'USO'* cultivars, 42.11-76.11 mg TE/g DW of seeds and blossoms *'Finola'* and *'USO'* cultivars, 17.18-21.89-mg TE/g DW of by-products, 14.88 mg TE/g DW of sample utilized for SCE-CO_2 optimization and, finally, 8.77-16.79 mg TE/g DW of stalks and roots (**Table 11**). The share of SCE-CO_2 extract towards these values was equal to 7% in *'Benico'* cultivar, on average 4.5% for leaves from *'Finola'* and *'USO'* cultivars, ~4.2% for seeds and blossoms for *'Finola'* and *'USO'* cultivars, ~8% for tested hemp by-products and 26.8% for sample, utilized for SCE-CO_2 optimization. After SCE-CO_2, $TEAC_{FRAP}$ values decreased in *'Benico'* sample by 4.8%, reaching 70.05 mg TE/ g DW of plant material before SCE-CO_2, in seeds and blossoms by 3.1-74.01% (29.89-53.67 mg TE/g DW prior SCE-CO_2), in leaves by 4.5-29.6% (46.82-67.16 mg TE/ g DW prior SCE-CO_2), and by 0.22%, 13.1%, 1.9-22.2% and 8.5-33.3 % in by-products, sample of unspecified *C. sativa* variety, stalks and roots. This study is in agreement with research results of Srivastava and Chauhan (2012), authors reported that *C. sativa* aqueous extracts of blossoms (74.8 $\mu MFe^{2+}/g$) and leaves (34.0 $\mu MFe^{2+}/g$) (unknown cultivar of *C. sativa* was extracted with Tris-HCl buffer at pH 7.0) have higher ferric reducing antioxidant power than stalks (14.5 $\mu MFe^{2+}/g$) and, generally, the same anatomical parts of fiber type hemp has up to 1.1-3.9 fold higher ferric reducing antioxidant power than other plants, like *Argemone mexicana, Datura metal, Calotropis procera, Thevetia peruviana* [98]. Also authors showed that ferric reducing antioxidant power depends on anatomical part of plant: blossoms (42.11-76.11 mg TE/g DW in *'Finola'* and *'USO'* cultivars) > leaves (56.83-80-59 mg TE/g DW in *'Finola'* and *'USO'* cultivars) > stalks (13.40-16.79 mg TE/g DW in *'Finola'* and *'USO'*

cultivars), which is in agrrement with the results of our study. Also, Mihoc and Pop (2013) reported that ferric reducing power of hemp seed oil is 80.73 μMFe^{+2}/g oil [193], which is similar to value of blossoms 74.8 μMFe^{2+}/g aqueous extract, reported by Srivastava and Chauhan (2012).

DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ are two most commonly applied, fast and relatively easy antiradical activity assessment assays [194], [169]. The DPPH$^{\bullet}$ is stable organic nitrogen radical, neutralized by radical quenching via H atom transfer or by direct reduction via electron transfer [155] [195]. ABTS$^{\bullet+}$ is ascribed to the electron transfer assays and dark-green coloured ABTS radical cation is obtained by oxidizing ABTS reagent with peroxyl radicals or other oxidants, e.g. potassium persulfate) [170, 194]. The increasing overall radical scavenging capacity in both assays is directly proportional to the decreasing absorbance values and expressed and Trolox equivalent antioxidant capacity. The DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging capacities of tested samples are listed in **Table 12**. The $TEAC_{DPPH}$ values ranged from 3.59 to 155.11 mg TE/g extract, with 63.28 mg TE/g for *'Benico'* sample, 9.5 mg TE/g for sample used for SCE-CO_2 optimization, 3.59-5.31 mg TE/g for by-products and with means of 107.25 and 50.3 mg TE/g across different vegetation period leaves, and seeds and blossoms, respectively. These results, expressed as mg TE/g DW, ranged from 0.29 to 4.75 mg TE/g DW with the following sequence of activity: *'Benico'* (4.75 mg TE/g DW) > leaves (mean of 2.9 mg TE/g DW) > seeds and blossoms (mean of 1.9 mg TE/g DW) > unspecified variety (1.96 mg TE/g DW) > by-products (mean of 0.54 mg TE/g DW). $TEAC_{DPPH}$ values of unextracted plant material (prior SCE-CO_2) amounted 3.06-41.88 mg TE/g DW, with '*Benico'* sample being most active (41.88 mg TE/g DW), followed by leaves (19.87-36.32 mg TE/g DW) and seeds and blossoms (21.13-31.37 mg TE/g DW) from '*Finola'* and '*USO'* cultivars. The share of SCE-CO_2 extract towards these properties was equal to 11% in *'Benico'* cultivar, and on average, 11 and 7.3% in leaves and seeds and blossoms from '*Finola'* and '*USO'* cultivars. DPPH$^{\bullet}$ scavenging capacity of these samples was up to 11.6 fold higher than of stalks (mean of 5.1 mg TE/g DW), roots (mean of 3.8 mg TE/g DW), unspecified *C. sativa* variety (6.72 mg TE/g DW) and hemp by- products (mean of 11.8 mg TE/g DW). The SCE-CO_2 reduced the radical scavenging activity of hemp by 0.29-23.5% in different anatomical parts, reaching 2.98-42.13 mg TE/g DW or 2.73-40.86 mg TE/g DW, when recalculated per gram of starting material before SCE-CO_2. In 2012 Uluata and Özedmir showed $TEAC_{DPPH}$ value of 62.37 mg TE/100 g oil, which is considerably lower as compared to our obtained result for *'Benico'* SCE-CO_2 extract (63.28 mg TE/g extract) [116, 117].

Table 12. DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging properties hemp samples before and after SCE-CO_2, and SCE-CO_2 extracts

Samples	$TEAC_{DPPH}$				$TEAC_{ABTS}$			
	SCE-CO_2 extracts		Plant material		SCE-CO_2 extracts		Plant material	
			Before SCE-CO_2	After SCE-CO_2			*Before SCE-CO_2*	*After SCE-CO_2*
	mg TE/g extract	*mg TE/g DW*	*mg TE/g DW*	*mg TE/g DW**	*mg TE/g extract*	*mg TE/g DW*	*mg TE/g DW*	*mg TE/g DW**
***Seeds and blossoms**:*								
'Finola' I veg.	34.44 ± 1.24^{c}	0.73 ± 0.03^{b}	21.13 ± 1.38^{ef}	25.03 ± 0.54^{fg}	741.53 ± 36.21^{e}	15.65 ± 0.76^{b}	114.35 ± 10.32^{c}	152.39 ± 18.07^{fg}
'Finola' II veg.	135.3 ± 3.61^{h}	3.98 ± 0.11^{g}	31.37 ± 1.84^{i}	37.03 ± 0.09^{h}	1038.71 ± 51.73^{g}	30.54 ± 1.52^{d}	194.82 ± 21.26^{g}	259.13 ± 17.69^{i}
'Finola' III veg.	25.54 ± 0.77^{b}	1.97 ± 0.06^{c}	24.81 ± 0.73^{g}	26.81 ± 1.02^{g}	632.30 ± 31.95^{d}	48.69 ± 2.46^{e}	139.51 ± 12.46^{de}	104.36 ± 6.29^{d}
'USO' III veg.	6.21 ± 0.23^{a}	0.92 ± 0.03^{b}	25.86 ± 0.69^{hg}	25.49 ± 1.31^{fg}	181.44 ± 24.50^{b}	26.91 ± 3.63^{c}	142.25 ± 10.45^{e}	116.98 ± 13.73^{de}
Leaves:								
'Finola' I veg.	155.11 ± 10.85^{i}	2.81 ± 0.20^{f}	22.81 ± 1.02^{fg}	24.35 ± 1.10^{f}	766.93 ± 59.88^{e}	13.88 ± 1.08^{b}	161.94 ± 9.00^{ef}	131.77 ± 13.73^{ef}
'Finola' II veg.	88.95 ± 0.54^{f}	2.22 ± 0.01^{e}	25.04 ± 0.30^{hg}	25.01 ± 0.88^{fg}	486.34 ± 18.17^{c}	12.16 ± 0.45^{b}	164.68 ± 14.15^{f}	116.73 ± 5.40^{de}
'Finola' III veg.	111.73 ± 1.63^{g}	3.96 ± 0.06^{g}	19.87 ± 0.76^{e}	21.79 ± 1.38^{e}	914.25 ± 19.55^{f}	32.36 ± 0.69^{d}	115.60 ± 7.47^{cd}	141.48 ± 7.86^{fg}
'USO' III veg.	73.22 ± 2.87^{e}	2.90 ± 0.11^{f}	36.32 ± 1.20^{j}	39.37 ± 0.77^{i}	702.16 ± 16.61^{ed}	27.81 ± 0.66^{cd}	156.70 ± 17.98^{e}	155.30 ± 13.01^{g}
Stalks:								
'Finola' I veg.	-^{na}	-^{na}	4.49 ± 0.04^{ab}	5.37 ± 0.07^{bc}	-^{na}	-^{na}	35.56 ± 1.30^{a}	33.63 ± 0.39^{ab}
'Finola' II veg.	-^{na}	-^{na}	5.55 ± 0.24^{bc}	5.54 ± 0.09^{bc}	-^{na}	-^{na}	37.65 ± 1.57^{a}	32.00 ± 1.42^{ab}
'Finola' III veg.	-^{na}	-^{na}	4.88 ± 0.37^{abc}	4.98 ± 0.12^{bc}	-^{na}	-^{na}	34.23 ± 3.50^{a}	29.33 ± 0.83^{ab}
'USO' III veg.	-^{na}	-^{na}	5.39 ± 0.30^{bc}	5.25 ± 0.08^{bc}	-^{na}	-^{na}	30.21 ± 2.79^{a}	31.45 ± 0.89^{ab}
Roots:								
'Finola' I veg.	-^{na}	-^{na}	4.05 ± 0.32^{ab}	3.83 ± 0.21^{ab}	-^{na}	-^{na}	34.88 ± 0.67^{a}	28.84 ± 2.52^{ab}
'Finola' II veg.	-^{na}	-^{na}	4.35 ± 0.13^{ab}	4.26 ± 0.10^{ab}	-^{na}	-^{na}	35.88 ± 1.26^{a}	30.54 ± 1.41^{ab}
'Finola' III veg.	-^{na}	-^{na}	3.68 ± 0.07^{ab}	2.99 ± 0.17^{a}	-^{na}	-^{na}	35.22 ± 2.31^{a}	26.63 ± 0.60^{ab}
'USO' III veg.	-^{na}	-^{na}	3.06 ± 0.08^{a}	2.98 ± 0.18^{a}	-^{na}	-^{na}	27.25 ± 1.59^{a}	23.75 ± 1.54^{a}
'Benico'**	63.28 ± 2.27^{d}	4.75 ± 0.17^{h}	41.88 ± 0.96^{k}	42.13 ± 0.84^{j}	1489.57 ± 40.38^{h}	111.87 ± 3.03^{f}	189.59 ± 9.26^{g}	235.36 ± 11.22^{h}
Unspecified variety***	9.05 ± 0.18^{a}	1.96 ± 0.04^{c}	6.72 ± 0.58^{c}	6.43 ± 0.48^{c}	108.68 ± 12.39^{ab}	23.49 ± 2.68^{c}	72.24 ± 7.95^{b}	63.85 ± 4.71^{c}
By-products I****	5.31 ± 0.17^{a}	0.29 ± 0.01^{a}	11.94 ± 0.71^{d}	9.04 ± 0.45^{d}	125.44 ± 21.15^{ab}	6.86 ± 1.16^{a}	36.12 ± 1.50^{a}	38.87 ± 1.25^{ab}
By-products II*****	3.59 ± 0.12^{a}	0.79 ± 0.03^{b}	11.67 ± 0.32^{d}	9.45 ± 0.80^{d}	66.77 ± 2.88^{a}	14.64 ± 0.63^{b}	78.80 ± 5.02^{b}	47.36 ± 2.79^{bc}

-^{na}: not available; *: mg TE/g sample after SCE-CO_2; **: mixture of seeds, blossoms and leaves; ***: C. sativa mixture of blossoms, leaves and Stalks; ****: C. sativa seed hulls (major part); *****- C. sativa seed hulls (minor part). Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$)

As given in **Table 12**, SCE-CO_2 extract activity in $ABTS^{\bullet+}$ assay was equal to 66.77-1489.57 mg TE/g extract. $TEAC_{ABTS}$ value of lipophilic constituents from *'Benico'* sample was 1.8-2.0 fold higher than the seeds and blossoms and leaves of other cultivars and significantly outnumbered (11.8-22.3 fold) the activity of hemp-by product and unspecified *C. sativa* variety SCE-CO_2 extracts. Taking into account the yield of extracts, these amounts are in the range of 6.86-111.87 mg TE/g DW plant material, maintaining above discussed order: *'Benico'* (111.87 mg TE/g DW) > seeds and blossoms (15.65-48.69 mg TE/g DW) from '*Finola'* and '*USO'* cultivars > leaves (12.16-32.36 mg TE/g DW) from '*Finola'* and '*USO'* cultivars > unspecified variety (23.49 mg TE/g DW) > by-products (6.86-14.64 mg TE/g DW). The share of SCE-CO_2 extract towards these properties was equal to 59% in *'Benico'* cultivar, on average, 14.4 and 20.6% in leaves and seeds and blossoms from '*Finola'* and '*USO'* cultivars, 32.5% in unspecified variety and on average 18.7 in by-products. After SCE-CO_2, $TEAC_{ABTS}$ values of solid residues of hemp samples were 23.75-235.36 mg TE/g DW and comprised on average 80% of the initial values, determined for the starting material before SCE-CO_2 (27.25-194.82 mg TE/g DW).

Oxygen radical absorbance capacity (ORAC) assay measures chain breaking antioxidant activity and substance capacity to inhibit peroxyl radical-induced oxidations via H atom transfer [196]. The advantage of ORAC assay against other radical scavenging assays is via the use of a biologically relevant radical source [172] and possibility to be applied for both for hydrophilic and lipophilic constituent activity evaluation [172,192]. Mechanism behind the assay: peroxyl radical reacts with a fluorescent probe and form a non-fluorescent product, which is quantified by fluorescence measurements. Antioxidant capacity is commonly expressed as Trolox equivalent antioxidant capacity. As presented in **Table 13**, $TEAC_{H\text{-}ORAC}$ values of extracts were equal to 28.89-898.05 mg TE/g SCE-CO_2 extract or 2.22-41.47 mg TE/g DW plant material and decreased in the following order: *'Benico'* (41.47 mg TE/g DW) > seeds and blossoms (8.81-26.39 mg TE/g DW) > leaves (8.50-18.65 mg TE/g DW) > by-products (2.68-5.05mg TE/g DW) > unspecified variety (2.22 mg TE/g DW). $TEAC_{H\text{-}ORAC}$ values of not extracted plant material showed the following trend: leaves (on average, 212.6 mg TE/g DW) > *'Benico'* (174.79 mg TE/g DW) > seeds and blossoms (on average, 130.3 mg TE/g DW) > by-products (on average, 38.4 mg TE/g DW) > stalks (on average, 36.05 mg TE/g DW) > roots (on average, 32.77 mg TE/g DW) > unspecified *C. sativa* variety (19.19 mg TE/g DW). The share of SCE-CO_2 extract towards these values was equal to 23.7% in *'Benico'* cultivar, ~5.45 and 13% in leaves and seeds and blossoms from '*Finola'* and '*USO'* cultivars, 11% in unspecified variety and on average 5.6 in by-products. The vast majority of solid residues after SCE-CO_2 are in the range of 28.85-235.79 mg TE/g DW, amounting aproximately 66.4% of the respective values, obtained for the starting material prior SCE-CO_2 (19.19-292.54 mg TE/g DW), and follows similar decreasing order: leaves (on average,

178.58 mg TE/g DW) > seeds and blossoms (on average, 150.59 mg TE/g DW) > *'Benico'* (142.06 mg TE/g DW) > by-products (on average, 38.92 mg TE/g DW) > roots (on average, 36.95 mg TE/g DW) > stalks (on average, 35.51 mg TE/g DW) > unspecified variety (24.74 mg TE/g DW). However, on the basis of our data 'Benico' cultivar distinguishes by the highest antioxidant scavenging properties among all other *C.sativa* cultivars tested

Table 13. H-ORAC oxygen radical scavenging capacity samples of hemp samples before and after SCE-CO_2, and SCE-CO_2 extracts, expressed as Trolox equivalent antioxidant capacity (TEAC, mg TE/g)

Samples	$TEAC_{H\text{-}ORAC}$			
	SCE-CO_2 extracts		Plant material	
			Before SCE-CO_2	After SCE-CO_2
	mg TE/g extract	*mg TE/g DW*	*mg TE/g DW*	*mg TE/g DW**
***Seeds and blossoms**:*				
'Finola' I veg.	572.98 ± 80.16^{c}	12.07 ± 1.69^{bc}	93.00 ± 9.66^{d}	157.73 ± 18.05^{de}
'Finola' II veg.	898.05 ± 102.01^{e}	26.39 ± 3.00^{d}	216.61 ± 13.68^{g}	235.79 ± 30.52^{g}
'Finola' III veg.	276.79 ± 26.54^{b}	21.31 ± 2.04^{d}	96.32 ± 6.73^{de}	78.16 ± 10.54^{b}
'USO' III veg.	114.44 ± 7.50^{a}	8.81 ± 0.58^{a}	115.14 ± 5.26^{e}	130.69 ± 15.64^{cd}
Leaves:				
'Finola' I veg.	799.69 ± 48.27^{de}	10.23 ± 7.14^{ab}	292.54 ± 18.04^{h}	109.81 ± 7.90^{c}
'Finola' II veg.	339.98 ± 34.92^{b}	8.50 ± 0.87^{ab}	231.73 ± 2.20^{g}	199.84 ± 23.19^{f}
'Finola' III veg.	532.86 ± 68.81^{c}	18.65 ± 2.41^{cd}	112.08 ± 3.16^{de}	169.99 ± 6.70^{ef}
'USO' III veg.	354.85 ± 23.44^{b}	9.23 ± 8.02^{ab}	214.27 ± 9.86^{g}	234.70 ± 16.12^{g}
Stalks:				
'Finola' I veg.	-^{na}	-^{na}	32.39 ± 2.83^{b}	28.85 ± 2.98^{a}
'Finola' II veg.	-^{na}	-^{na}	35.20 ± 3.97^{b}	39.75 ± 3.67^{a}
'Finola' III veg.	-^{na}	-^{na}	37.93 ± 0.20^{b}	33.49 ± 4.35^{a}
'USO' III veg.	-^{na}	-^{na}	38.68 ± 5.06^{b}	39.95 ± 3.40^{a}
Roots:				
'Finola' I veg.	-^{na}	-^{na}	34.53 ± 1.19^{b}	34.52 ± 2.22^{a}
'Finola' II veg.	-^{na}	-^{na}	30.63 ± 3.29^{b}	41.95 ± 2.06^{a}
'Finola' III veg.	-^{na}	-^{na}	27.88 ± 0.29^{ab}	41.48 ± 1.52^{a}
'USO' III veg.	-^{na}	-^{na}	38.06 ± 2.20^{b}	29.85 ± 2.62^{a}
'Benico'*	758.06 ± 58.01^{d}	41.47 ± 0.27^{e}	174.79 ± 21.36^{f}	142.06 ± 11.59^{de}
Unspecified variety **	28.89 ± 0.78^{a}	2.22 ± 0.06^{a}	19.19 ± 2.93^{ab}	24.74 ± 2.81^{a}
By-products I***	35.98 ± 3.50^{a}	2.68 ± 0.27^{a}	67.28 ± 0.59^{a}	53.10 ± 9.6^{ab}
By-products II****	68.72 ± 5.77^{a}	5.05 ± 0.44^{ab}	69.49 ± 9.39^{c}	34.06 ± 6.88^{a}

-^{na}: not available; *: mg TE/g sample after SCE-CO_2; **: mixture of seeds, blossoms and leaves; ***: C. sativa mixture of blossoms, leaves and Stalks; ****: C. sativa seed hulls (major part); *****: C. sativa seed hulls (minor part). Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$)

Summarizing results in **Table 11**, **Table 12**, **Table 13**, *C. sativa 'Benico'* cultivar shows higher antioxidant capacity as compared to *'Finola'* and *'USO'* cultivars. In addition, these properties significantly vary in different anatomical plant parts and plant vegetation period. Seeds

corresponding anatomical parts of *III* vegetation. The antioxidant properties of hemp stalks and roots are considerably (9-12 fold) lower, as compared to the seeds and leaves. Also, considerable amount of bioactive compounds are polar compounds and remains in the solid residues after SCE-CO_2 [194].

3.2. Development of 4-step biorefining protocol for cannabinoid and antioxidant fraction isolation from *C. sativa 'Benico'* cultivar

For the purposes of this research and further biorefining protocol development to isolate valuable cannabinoid and antioxidant fractions (**Fig. 12**), mixture of seeds, blossoms and leaves of *C. sativa 'Benico'* cultivar was selected.

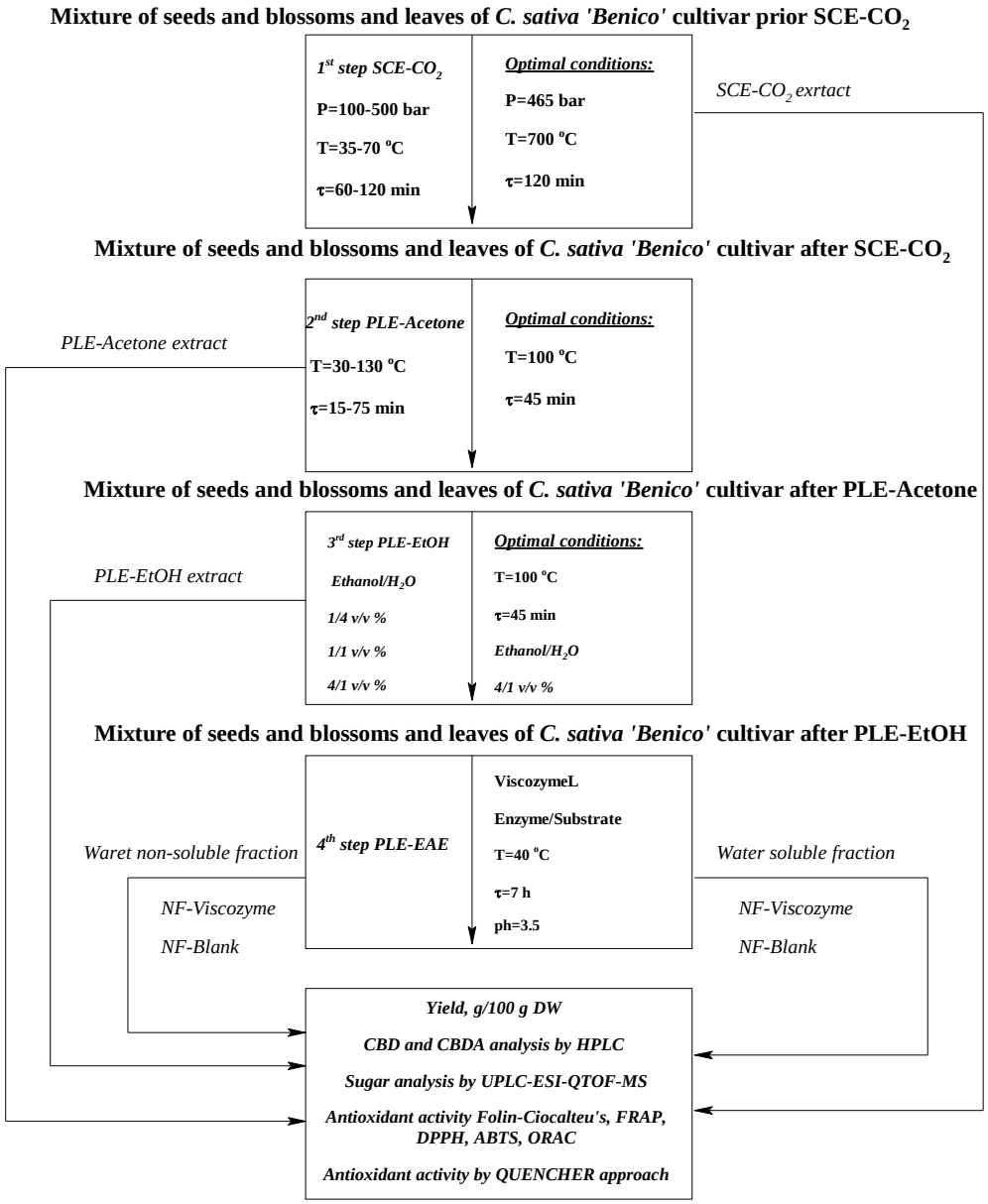


Fig. 12. Schematic representation of 4-step biorefining process by high pressure fractionation and enzyme-assisted extraction of *S. sativa 'Benico'* cultivar, and analysis of various fractions obtained

C. sativa 'Benico' cultivar distinguished among other samples tested by: (1) high content of CBD and CBDA, as recalculated per gram of DW plant material (**Table 10**); (2) the highest efficiency of SCE-CO_2 to concentrate these cannabinoids (99% for CBD and 88% for CBDA) in lipophilic fraction (**Table 10**); (3) highest total phenolic content, *in vitro* radical scavenging

capacity and reducing power for SCE-CO_2 extracts and plant material before and after extraction (**Table 11**, **Table 12**, **Table 13**). As depicted in **Fig. 12**, fractionation of this selected *C. sativa* cultivar was performed sequentially employing high pressure (SCE-CO_2 and PLE) and enzyme-assisted (EAE) extraction techniques.

3.2.1. Optimisation of SCE-CO_2 parameters for 'Benico' cultivar

For SCE-CO_2 optimization, mixture of blossoms and leaves, originating from '*Benico'* cultivar, was utilised. Central composite design (CCD) of 20 experimental runs (8 factorial points, 6 axial points and 6 centre points (**Table 14**) and response surface methodology (RSM) were used to optimize the effect of three independent variables on the SCE-CO_2 extract and cannabinoid (CBD and CBDA) yields: pressure (P, 100-500 bar), temperature (T, 35-70°C) and extraction time (τ, 60-120 min). Additional SCE-CO_2 was implemented due to differences in plant material anatomical and chemical composition, previously published literature data on SCE-CO_2 process optimization was mainly focused hemp seed oil yields, with no reports on extraction parameter optimization to isolate and concentrate target bioactive constituents, e.g. cannabinoids, from various hemp cultivars and plant anatomical parts [119].

3.2.1.1.Model analysis for SCE-CO_2 extract yield

As presented in **Table 14**, experimentally obtained lipophilic fraction yields were from 0.27 to 10.36 g/100 g DW of *C. sativa* cultivar '*Benico'*. The adequacy of the model, evaluated by the total determination coefficient R^2 (value of 0.9979), indicated a reasonable fit of the model to the experimental data. Adjusted coefficient of determination (R^2) of 0.9960 was in agreement with the predicted coefficient of determination (R^2) of 0.9763. Model evaluation is presented in the analysis of variance (**Table 15**). The significance of each model was determined using the Student test (p-value). The analysis of the quadratic regression models for extract yield showed that the model was significant ($p < 0.05$) with an F-value of 3.35 and the *'lack of fit'* was not significant relative to the pure error, with a p-value of 0.1053. The model shows that the factor with the extraction pressure (P), temperature (T) and time (τ) ($p < 0.05$) are significant for yield. Interaction between pressure and temperature (PT) is significant ($p < 0.05$). The second-order term pressure (P^2) was significant ($p < 0.05$).

Table 14. *C. sativa 'Benico'* cultivar SCE-CO_2 parameters optimisation and CBD and CBDA amount in extracts obtained by SCE-CO_2

No.	SCE-CO_2 parameters			Yield	CBD		CBDA	
	Pressure, bar	*Temperature, °C*	*Time, min*	*g/100 g DW*	*mg/g extract*	*g/100 g DW*	*mg/g extract*	*g/100 g DW*
1	100	35	60	1.76 ± 0.03^{b}	64.181 ± 2.979^{e}	0.113 ± 0.005^{ab}	157.561 ± 6.002^{a}	0.277 ± 0.011^{a}
2	300	70	90	7.93 ± 0.08^{cd}	25.750 ± 1.305^{d}	0.204 ± 0.001^{ef}	185.601 ± 8.258^{ab}	1.472 ± 0.06^{bc}
3	300	52.5	90	7.62 ± 0.09^{cd}	19.132 ± 0.725^{ab}	0.146 ± 0.006^{bcd}	232.552 ± 0.488^{bcd}	1.772 ± 0.004^{de}
4	100	52.5	90	0.63 ± 0.00^{a}	-nd	-nd	-nd	-nd
5	300	52.5	60	7.65 ± 0.08^{cd}	18.307 ± 0.717^{a}	0.140 ± 0.005^{abc}	236.296 ± 14.007^{bcd}	1.808 ± 0.107^{de}
6	100	70	120	0.27 ± 0.01^{a}	-nd	-nd	-nd	-nd
7	300	52.5	90	7.90 ± 0.13^{cd}	18.461 ± 3.614^{ab}	0.146 ± 0.028^{bcd}	231.550 ± 9.910^{bcd}	1.829 ± 0.076^{de}
8	300	52.5	90	7.61 ± 0.05^{cd}	21.002 ± 0.725^{abcd}	0.160 ± 0.006^{cd}	233.272 ± 1.507^{bcd}	1.775 ± 0.011^{de}
9	100	70	60	$< 0.00^{a}$	-nd	-nd	-nd	-nd
10	300	52.5	90	7.56 ± 0.00^{cd}	20.894 ± 0.174^{abcd}	0.158 ± 0.001^{c}	237.800 ± 3.108^{cd}	1.799 ± 0.024^{de}
11	500	52.5	90	8.25 ± 0.19^{d}	21.412 ± 0.21^{abcd}	0.177 ± 0.002^{de}	231.464 ± 2.441^{bcd}	1.909 ± 0.020^{ef}
12	100	35	120	2.51 ± 0.02^{b}	44.223 ± 1.940^{e}	0.111 ± 0.005^{a}	203.817 ± 7.875^{b}	0.512 ± 0.022^{a}
13	500	70	60	9.63 ± 0.26^{e}	24.154 ± 0.891^{bcd}	0.233 ± 0.009^{f}	204.045 ± 7.302^{b}	1.965 ± 0.070^{ef}
14	300	52.5	120	7.80 ± 0.17^{cd}	20.158 ± 0.958^{abcd}	0.157 ± 0.007^{cd}	221.115 ± 12.481^{bc}	1.725 ± 0.096^{cde}
15	300	35	90	7.30 ± 0.13^{c}	23.802 ± 0.542^{bcd}	0.174 ± 0.004^{de}	236.231 ± 12.153^{bcd}	1.724 ± 0.089^{cde}
16	500	70	120	10.36 ± 0.31^{e}	21.524 ± 0.842^{abcd}	0.223 ± 0.009^{f}	223.646 ± 14.108^{bc}	2.317 ± 0.149^{f}
17	300	52.5	90	7.50 ± 0.16^{cd}	20.894 ± 0.174^{abcd}	0.157 ± 0.001^{cd}	239.278 ± 1.018^{cd}	1.795 ± 0.008^{de}
18	500	35	60	7.39 ± 0.21^{cd}	19.416 ± 0.890^{abc}	0.144 ± 0.007^{abcd}	179.895 ± 9.223^{ab}	1.330 ± 0.068^{b}
19	300	52.5	90	7.63 ± 0.05^{cd}	20.067 ± 0.597^{abc}	0.153 ± 0.005^{cd}	230.418 ± 9.910^{bc}	1.758 ± 0.076^{de}
20	500	35	120	7.51 ± 0.04^{cd}	18.277 ± 0.681^{a}	0.137 ± 0.005^{abc}	209.136 ± 7.556^{b}	$1.572 \pm 0.057b^{cd}$
					Optimal conditions:			
	465	**70**	**120**	**8.30 ± 0.01^{d}**	**24.721 ± 0.314^{cd}**	**0.205 ± 0.003^{ef}**	**261.400 ± 2.243^{d}**	**2.169 ± 0.019^{f}**

-nd: not detected. Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$)

Table 15. Analysis of variance table for response surface quadratic model for SCE-CO_2 extract yield (g/100 g DW), isolated from *C. sativa* cultivar *'Benico'*

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Response factor I: SCE-CO_2 extract yield (g/100 g DW)					
Model	195.26	9	21.70	525.26	< 0.0001*
Pressure (P, bar)	144.17	1	144.17	3490.40	< 0.0001*
Temperature (T, °C)	0.30	1	0.30	7.16	0.0232*
Time (τ, min)	0.41	1	0.41	9.88	0.0105*
PT	10.33	1	10.33	250.05	< 0.0001*
Aτ	$3.613 \cdot 10^{-3}$	1	$3.613 \cdot 10^{-3}$	0.087	0.7735**
Tτ	$2.113 \cdot 10^{-3}$	1	$2.113 \cdot 10^{-3}$	0.051	0.8256**
P^2	25.47	1	25.47	616.57	< 0.0001*
T^2	0.048	1	0.048	1.16	0.3074**
τ^2	0.16	1	0.16	3.89	0.0767**
Residual	0.41	10	0.041		
Lack of fit	0.32	5	0.064	3.35	0.1053**
Pure error	0.095	5	0.019		
Corrected Total	195.68	19			

*: significant; **: not significant

Predicted values were calculated using a second order polynomial equation and compared with experimental values in **Fig. 13**. Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is given in the following equation:

$$Yield, \% = 7.58 + 3.80 \times P + 0.17 \times T + 0.20 \times \tau + 1.14 \times P \times T + 0.021 \times P \times \tau - 3.04 \times P^2 + 0.13 \times T^2 + 0.24 \times \tau^2$$

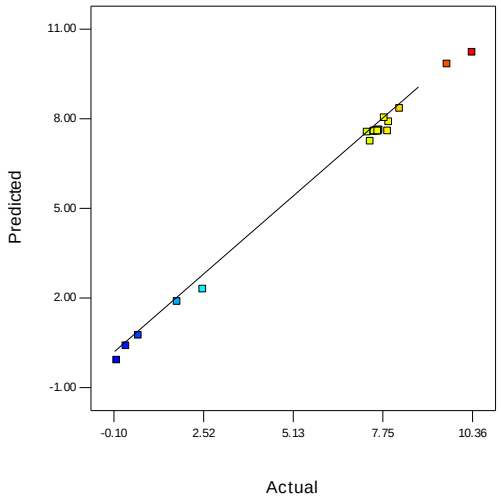


Fig. 13. Comparison of actual and predicted SCE-CO_2 extract yield from *C. sativa* cultivar *'Benico*

Response surface plots showing the effect of extraction pressure, temperature, and time on extract yield are presented in **Fig. 14**. **Fig. 14** (A) illustrates the effect of extraction temperature and pressure on extract yield at constant 90 min extraction time. In this case, extraction pressure

has significant influence on extract yield, while changing of extraction temperature no significant alterations (increase or decrease) is obtained. **Fig. 14** (B) demonstrates that the yield increases by increasing pressure at constant temperature of 52.5°C. In this case extraction pressure exerted a strong quadratic effect and time had slightly positive effect on extract yield. It was also determined that continuation of extraction after 90 min process time had negligible effect on the extract yield increase [182]. **Fig. 14** (C) demonstrates that the yield increases by increasing time at constant pressure of 300 bar. It was also determined that continuation of extraction after 90 min has negligible effect on the extract yield.

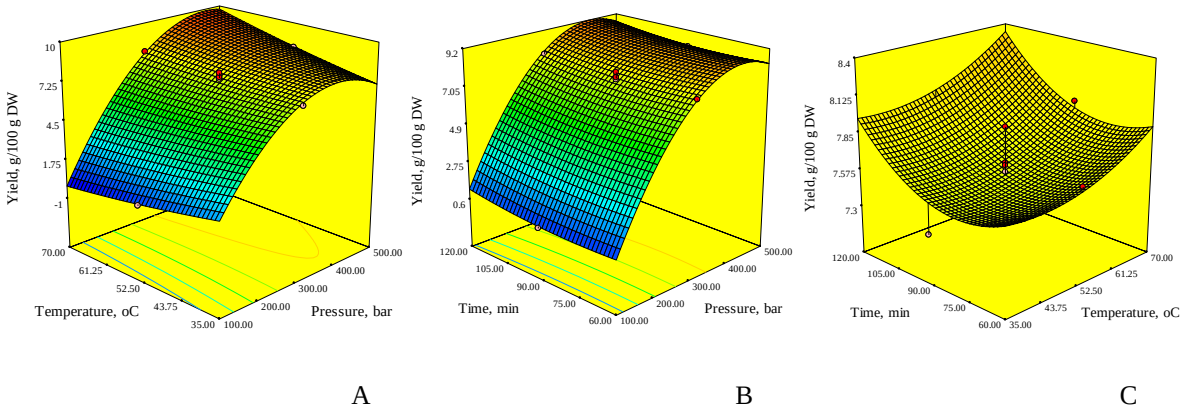


Fig. 14. 3D RSM plots of the dependencies of hemp extract yield on temperature/pressure (A), time/pressure (B) and time/temperature (C)

Considering all responses, optimal conditions for obtaining the highest SCE-CO_2 extract yield (8.30 g/100 g) from mixture of seeds, blossoms and leaves of *C. sativa 'Benico'* cultivar were 120 min extraction time, 70°C temperature and 465 bar pressure. For '*Benico*' cultivar optimal SCE-CO_2 conditions and effect of independent variables slightly differ from those, obtained for a mixture of seeds, stems and leaves from unspecified *C. sativa* cultivar (Chapter 3.1.1): in this case all variables and interaction between pressure and temperature are significant, while only extraction pressure was found to be significant in the first attempt to optimize SCE-CO_2 parameters. Also, higher selected optimal pressure and temperature increases SCE-CO_2 extract yield for *'Benico'* cultivar by 9.5 %, as compared to data listed in **Table 14**.

3.2.1.2.Model analysis for CBD and CBDA yields

According to the experimental **Table 14** CBD content in *'Benico'* SCE-CO_2 extracts under different experimental extraction conditions varied from 18.277 to 64.181 mg/g extract (0.111-0.233 mg/g DW). Experimentally obtained CBDA amounted from 157.561 to 239.278 mg/g extract (0.277-2.317 g/100 g DW). Model evaluations for response factors II (CBD yield) and III (CBDA yield) are presented in **Table 16** and **Table 17**, respectively. Looking at the results for CBD extraction (, the analysis of the quadratic regression models showed that the suggested model

was significant ($p < 0.05$) with an F-value of 9.91 and the "lack of fit" was significant relative to the pure error, with a p-value of 0.0125. Extraction pressure (P) ($p < 0.05$) is significant positive effect on CBD yield. Interaction between pressure and temperature (PT) and second-order terms pressure (P^2) and temperature (T^2) were significant too, with $p < 0.05$. The adequacy of the model for CBD was evaluated by the total determination coefficient (R^2) value of 0.9756, indicating a reasonable fit of the model to the experimental data. Adjusted coefficient of determination (R^2) was equal to 0.9537, predicted coefficient of determination (R^2) – to 0.8247.

Table 16. Analysis of variance table for response surface quadratic model for CBD yield (g/100 g DW), isolated from *C. sativa* cultivar *'Benico'*

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Response factor II: CBD yield (g/100 g DW)					
Model	0.082	9	$9.092 \cdot 10^{-3}$	44.47	< 0.0001*
Pressure (P, bar)	0.048	1	0.048	232.86	< 0.0001*
Temperature (T, °C)	$3.610 \cdot 10^{-5}$	1	$3.610 \cdot 10^{-5}$	0.18	0.6832**
Time (τ, min)	$4.000 \cdot 10^{-7}$	1	$4.000 \cdot 10^{-7}$	$1.956 \cdot 10^{-3}$	0.9656**
PT	0.020	1	0.020	97.33	< 0.0001*
Aτ	$2.813 \cdot 10^{-5}$	1	$2.813 \cdot 10^{-5}$	0.14	0.7185**
Tτ	$1.250 \cdot 10^{-7}$	1	$1.250 \cdot 10^{-7}$	$6.114 \cdot 10^{-4}$	0.9808**
P^2	0.011	1	0.011	56.03	< 0.0001*
T^2	$3.555 \cdot 10^{-3}$	1	$3.555 \cdot 10^{-3}$	17.39	0.0019*
τ^2	$5.682 \cdot 10^{-5}$	1	$5.682 \cdot 10^{-5}$	0.28	0.6096**
Residual	$2.045 \cdot 10^{-3}$	10	$2.045 \cdot 10^{-4}$		
Lack of fit	$1.857 \cdot 10^{-3}$	5	$3.715 \cdot 10^{-4}$	9.91	0.0125*
Pure error	$1.873 \cdot 10^{-4}$	5	$3.747 \cdot 10^{-5}$		
Corrected Total	0.084	19			

*: significant; **: not significant

CBDA model analysis (**Table 17**): regression models showed that the model was significant ($p < 0.05$) with an F-value of 56.38 and the *'lack of fit'* was significant relative to the pure error, with a p-value of 0.0002. In agreement to CBD model results, factors with the largest effect on CBDA yield were: (1) extraction pressure (P) ($p < 0.05$); (2) interaction between pressure and temperature (PT) is significant ($p < 0.05$) and (3) the second-order term pressure (P^2) ($p < 0.05$). The total determination coefficient was equal to 0.9829 (reasonable fit of the model to the experimental data), adjusted coefficient of determination – to 0.9675 and predicted coefficient of determination – to 0.8563. In comparison with previously described extract yield optimisation, her only pressure is significant for CBD yield, while in first optimisation pressure and temperature were significant.

Table 17. Analysis of variance table for response surface quadratic model for CBDA yield (g/100 g DW), isolated from *C. sativa* cultivar *'Benico'*

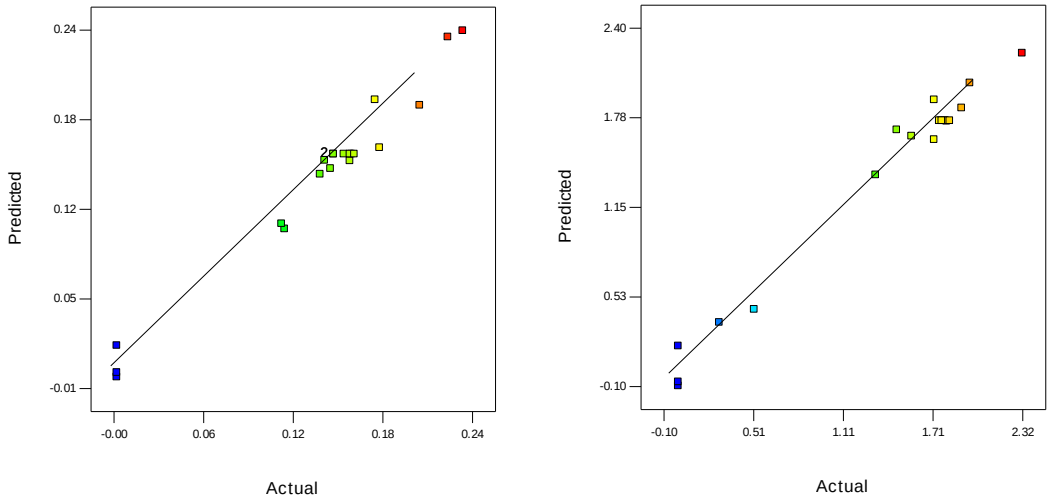
Source	Sum of Squares	df	Mean Square	F-Value	p-value
Response factor III: CBDA yield (g/100 g DW)					
Model	10.47	9	1.16	63.82	< 0.0001*
Pressure (P, bar)	6.90	1	6.90	378.38	< 0.0001*
Temperature (T, °C)	0.011	1	0.011	0.63	0.4456**
Time (τ, min)	0.056	1	0.056	3.05	0.1111**
PT	0.59	1	0.59	32.27	0.0002*
Aτ	0.016	1	0.016	0.88	0.3693**
Tτ	$1.953 \cdot 10^{-3}$	1	$1.953 \cdot 10^{-3}$	0.11	0.7501**
P^2	1.51	1	1.51	82.92	< 0.0001*
T^2	0.026	1	0.026	1.44	0.2574**
τ^2	0.014	1	0.014	0.75	0.4053**
Residual	0.18	10	0.018		
Lack of fit	0.18	5	0.036	56.38	0.0002*
Pure error	$3.176 \cdot 10^{-3}$	5	$6.352 \cdot 10^{-4}$		
Corrected Total	10.65	19			

*: significant; **: not significant

The following second order polynomial regressions were calculated for CBD and CBDA yields and compared to experimental yields in **Fig. 15**:

$$CBD\ yield, \% = 0.15 + 0.069 \times P - 0.0019 \times T - 0.0002 \times \tau + 0.05 \times P \times T - 0.001875 \times P \times \tau - 0.0000125 \times T \times \tau - 0.065 \times P^2 + 0.036 \times T^2 + 0.004545 \times \tau^2$$

$$CBDA\ yield, \% = 1.75 + 0.38 \times P + 0.034 \times T + 0.075 \times \tau + 0.27 \times P \times T + 0.045 \times P \times \tau - 0.016 \times T \times \tau - 0.74 \times P^2 - 0.098 \times T^2 + 0.071 \times \tau^2$$



A B

Fig. 15. Comparison of actual and predicted CBD (A) and CBDA (B) yield (g/100 g DW) values from *C. sativa* cultivar *'Benico'*

Response surface plots obtained by RSM, depicting the effects of SCE-CO_2 parameters on the CBD and CBDA yields, are presented in **Fig. 16** and **Fig. 17** respectively. **Fig. 16** (A) illustrates the effect of extraction temperature and pressure on CBD yield at constant 90 min extraction time:

extraction pressure up to 400 bar has significant influence on CBD content, higher pressure (more than 400 bar) has negative effect on CBD amount. **Fig. 16** (B) demonstrates that the CBD amount increases by increasing pressure at constant temperature of 52.5°C, while extraction time is not important on CBD content in g/100 g DW. Similarly, **Fig. 16** (C) demonstrates that the time has no importance on CBD content at constant pressure of 300 bar. Similarly to CBD model results, extraction pressure has significant influence on CBDA yield too, with no significant effect of temperature observed. (**Fig. 17**, (A) time 90 min). CBDA yield increases by increasing pressure at constant temperature of 52.5°C and in this case time has slightly positive effect on extract yield (**Fig. 17**, B). **Fig. 17** (C) demonstrates that the yield increases by increasing time at constant pressure of 300 bar and increasing of temperature has some positive effect on the extract yield.

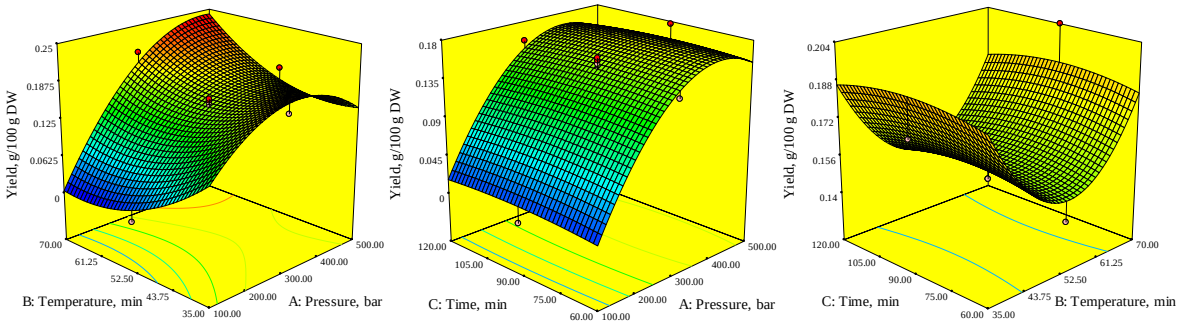


Fig. 16. 3D RSM plots of the dependencies of CBD yield on extraction temperature/pressure (A), time/pressure (B) and time/temperature (C)

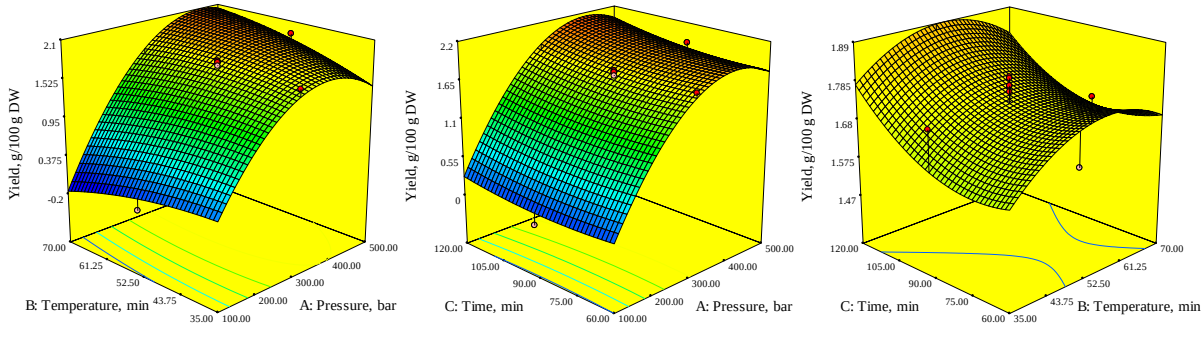


Fig. 17. 3D RSM plots of the dependencies of CBDA content on temperature/pressure (A), time/pressure (B) and time/temperature (C)

Considering all responses, the following optimal conditions to extract the highest CBD amount were: 450-460 bar, 70°C and 120 min. Under these conditions, a mixture of seeds, blossoms and leaves from *C. sativa* cultivar '*Benico*' yields 24.721 mg of CBD per gram of SCE-CO_2 extract, equal to 0.205 g of CBD per 100 g DW of plant material before extraction. The optimal conditions for CBDA extraction are the following: 450-460 bar, 70°C and 120 min, yielding 261.4 mg /g extract or 2.169 g/100 g DW of starting material. Comparing optimal

SCE-CO_2 extraction conditions for response factors I (SCE-CO2 extract yield), II (CBD yield) and III (CBDA yield), could be concluded that optimal conditions (for the highest extract yield) could by also applied for CBD and CBDA isolation and concentration in SCE-CO_2 lipophilic fraction.

3.2.2. Optimisation of PLE parameters for Benico cultivar

As was shown in Chapter 3.1, remaining plant material after SCE-CO_2 could be bio-refined to isolate higher polarity hemp compounds with potential antioxidant capacity. Due to these reasons, *C. sativa* cultivar '*Benico*' sample residue after SCE-CO_2 was further subjected for pressurized liquid extraction (PLE), sequentially using acetone and ethanol/water mixture (**Fig. 8**). PLE is the alternative method for different hydrophilic compounds (e.g. phenolic acids, flavonoids and polyphenols) extraction from plant material. This technique typically uses solvents, such as methanol, acetone, ethanol and water, and functions at temperatures above solvents boiling temperature. High pressure is used to keep the solvents in liquid form, while the extraction is ongoing. [136]. For the purposes of this research, optimization of PLE consisted of two stages: (1) optimization of extraction temperature (30-130°C) and time (15-70 min), when acetone was used as a solvent (**Table 18**) and (2) selection of ethanol/water ratio (EtOH/H_2O, 1:4-4:1 v/v) for further extraction of plant residue after PLE-Acetone (**Table 19**). The efficiency of extraction was evaluated by yield and TPC content of acetone and EtOH/H_2O extracts and plant material after PLE under different experimental conditions.

Table 18. Optimization of PLE-Acetone conditions for *'Benico'* residue after SCE-CO_2

Extraction parameters	Yield	TPC		
		Extracts		Plant material after PLE-Acetone
	g/100 g DW	*mg GAE/g extract*	*mg GAE/g DW*	*mg GAE/g DW**
15 min (3×5)				
30 °C	0.66 ± 0.13^{a}	92.77 ± 3.31^{a}	0.61 ± 0.02^{a}	34.16 ± 2.05^{e}
70 °C	1.79 ± 0.25^{c}	123.00 ± 0.84^{cd}	2.20 ± 0.01^{c}	32.41 ± 2.02^{e}
100°C	3.45 ± 0.07^{e}	131.11 ± 10.48^{e}	4.45 ± 0.36^{f}	32.01 ± 0.33^{de}
130°C	6.61 ± 0.18^{g}	152.16 ± 4.12^{h}	10.05 ± 0.27^{h}	32.10 ± 0.96^{e}
45 min (3×15)				
30 °C	1.20 ± 0.00^{b}	100.84 ± 2.50^{b}	1.20 ± 0.03^{b}	31.22 ± 1.08^{cd}
70 °C	3.36 ± 0.06^{de}	120.40 ± 5.45^{bc}	3.98 ± 0.18^{e}	24.04 ± 1.99^{ab}
100 °C	5.76 ± 0.06^{f}	138.82 ± 3.77^{f}	7.94 ± 0.22^{c}	29.52 ± 1.16^{c}
130 °C	7.74 ± 0.08^{h}	148.30 ± 5.43^{g}	11.40 ± 0.42^{i}	25.00 ± 4.44^{b}
75 min (3×25)				
70 °C	3.00 ± 0.00^{d}	97.16 ± 6.45^{b}	2.90 ± 0.20^{d}	23.76 ± 1.08^{a}
100 °C	5.46 ± 0.20^{f}	125.58 ± 8.57^{d}	6.67 ± 0.50^{g}	25.00 ± 0.35^{b}

*: mg GAE/g DW of sample after SCE-CO_2; Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$)

Table 19. Optimization of PLE-EtOH/H_2O conditions for *'Benico'* residue after PLE-Acetone

Extraction parameters	Yield	TPC		
		Extracts		Plant material
	g/100 g DW	*mg GAE/g extract*	*mg GAE/g DW*	*mg GAE/g DW**
100 °C, 45 min (3×15)				
Ethanol/H_2O, 1/4 v/v %	35.05 ± 0.78^{b}	83.04 ± 5.23^{a}	29.10 ± 1.83^{b}	7.46 ± 0.53^{b}
Ethanol/H_2O, 1/1 v/v %	20.71 ± 0.44^{a}	124.96 ± 3.67^{b}	25.88 ± 0.76^{a}	5.26 ± 0.46^{a}
Ethanol/H_2O, 4/1 v/v %	22.66 ± 1.54^{a}	132.77 ± 1.32^{b}	30.10 ± 0.30^{b}	10.59 ± 0.82^{c}

*: mg GAE/g DWof sample after SCE-CO_2; Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$)

As reported in **Table 18**, after 15 min of extraction at 30-130°C yield of acetone extract was in the range of 0.66-6.6 g/100 g DW, with TPC values of 92.77-152.16 mg GAE/g extract. The highest amount of this fraction (6.6 g/100 g DW) was collected at 130°C temperature. After 45 min of extraction yield varied from 1.20 to 7.74 g/100 g DW, TPC values – from 100.84 to 148.30 mg GAE/g extract) and again 130°C temperature yielded the highest amount of acetone fraction (7.74 g/100 g DW). The efficiency of PLE at the longest extraction time (75 min) was evaluated only under 70 and 100°C due to the following reasons: (1) generally, yields obtained at 30°C were negligible; (1) extracts, isolated at 130°C were characterized by faded yellow colour and noticeable sweet smell, which could be attributed to the degradation and/or unfavourable chemical interactions between various endogenous extract constituents, e.g. via the Maillard reaction pathway [197]. Therefore after 75 min of extraction at 70 and 100°C, 2.994 and 5.315 g of acetone extract per 100 g of plant material were obtained, respectively, with higher TPC activity measured for sample at 100°C. Nevertheless, the longest extraction time (75 min) was noticed to yield up to 12% lower amount of extract with up to 30% lower TPC values as compared to shorter treatments (45 min). Based on these results, the selected optimal PLE-Acetone conditions were: temperature 100°C, time 45 min.

The determined optimal temperature and extraction time for PLE-Acetone were further applied to select the best ethanol/water ratio for PLE-EtOH/H_2O (**Table 19**). Looking at the obtained results, the highest yield (34.05 g/100 g DW) was extracted using EtOH/H_2O at the ratio of 1/4 v/v %, however the highest concentration of ethanol in mixture yielded extracts with up to 38% higher TPC values. Therefore, the highest EtOH/H_2O ratio (4/1 v/v %) was selected for PLE-EtOH/H_2O at 100°C for 45 min. In studies reported by Chen and He (2012) extraction with ethanol and ultrasonic bath 40 kHz for 30 min was applied for for *C. sativa 'Bama'* and *'Yunma'* cultivars. Authors showed that higher ethanol concentration in EtOH/H_2O mixture yielded lower amount of extract: 100% (on average 1.435%) < 75% (on average 8.44%) < 50% (on average 10.56%), Our results are in agreement with these findings and additionally show 2.14 fold higher yield and PLE-EtOH/H_2O efficiency for polar fraction isolation from hemp [8].

3.2.3. Enzyme-assisted extraction

As presented in 4-step biorefining protocol **Fig. 8**, *C. sativa* cultivar '*Benico*' sample residue after optimized PLE-EtOH/H_2O was further utilized for enzyme-assisted extraction (EAE) experiments with commercially available cellulolytic enzyme mixture Viscozyme ®L. Viscozyme ®L is a complex multi-enzyme, which contains a wide range of carbohydrases (arabanase, cellulase, βglucanase, hemicellulase and xylanase), which breaks down glycosidic connections and is successfully applied for valuable nutrient (sugars, pectins), essential oil and plant polyphenol constituent extraction from various plant materials and by-products at both laboratory and industrial scale. Also it was noticed that Viscozyme® L reduces the viscosity of plant materials by degrading polysaccharides into lower molecular weight saccharides or disaccharides and thus enhance extraction efficiency thereof [137, 138, 198]. This is one of the most important factors for biomass saccharification, because the economy of bio-refineries depend on the several factors, such as sugar yield, processing costs, capital investments, and so on [199]. In addition, extractabilty of bioactive hep constituent could be increased by enzymatic treatment and release of cell-wall bound bioactive phenolics from matrix [139].

As previously shown by Vuorela and Meyer (2003), the optimal conditions for Viscozyme® L are the following: pH range of 3.3-5.5 and temperature of 25-55 °C [198]. For the purposes of this research, EAE was conducted under the following conditions: enzyme/substrate ratio 6% (corresponds to 72 FBGU/10 g of residue after PLE-EtOH/H_2O), temperature 40°C, pH 3.5, extraction time 7 hours. This EAE-Viscozyme treatment yielded 29.48 g/100 g DW of residual plant material after PLE- EtOH/H_2O (**Table 20**). This amount was significantly (~2 fold) higher as compared to the corresponding control sample (no enzyme added), yielding only 13.70 g of EAE-extract/100 g DW.

Table 20. Hexose and dihexose quantifiaction in water-soluble fraction and control sample after EAE (E/S 6% v/w, 40°C, pH 3.5, 7 hours) from *C. sativa 'Benico*' cultivar

Compound	**Viscozyme L**		**Blank**	
	mg /g extract	*g /100g DW*	*mg /g extract*	*g /100 g DW*
Yield:		29.48 ± 0.33		13.70 ± 0.52
Hexoses, mg GLU/g:				
Glucose*	77.62 ± 1.79^{c}	2.29 ± 0.05^{b}	9.36 ± 0.10^{a}	0.13 ± 0.00^{a}
Hexose**	86.11 ± 6.5^{c}	2.54 ± 0.19^{b}	61.17 ± 1.84^{d}	0.84 ± 0.30c
Hexose**	277.10 ± 8.32^{d}	8.17 ± 0.25^{c}	15.61 ± 1.08^{b}	0.21 ± 0.01^{ab}
Dihexoses, mg MAU/g:				
Maltose*	17.53 ± 0.50^{b}	0.52 ± 0.02^{a}	-nd	-nd
Dihexose**	2.52 ± 0.42^{a}	0.07 ± 0.01^{a}	26.98 ± 0.85^{c}	0.37 ± 0.02^{b}
Dihexose**	5.84 ±0.87^{b}	0.17 ± 0.03^{a}	-nd	-nd

-nd: not detected; *: confirmed by standard; **: confirmed by m/z value of 178.8 and 341.0 for hexoses and dihexoses; GLU: glucose units; MAU: maltose units. Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$)

Hemp plants are characterized by relatively high carbohydrates such as glucose (~83%), cellulose (~18%) and others [10, 11]. Therefore, the efficiency of EAE-Viscozyme treatment was

additionally assessed the amount of hexoses and dihexoses in supernatants. In total, these constituents comprised respectively 13.00 and 0.76 g/100 g DW in EAE-Viscozyme extract and respectively 1.80 and 0.37 g/100 g DW in control sample. Looking at the individual constituent profiles, the content of extracted glucose from the Viscozyme-treated sample was by 94% higher as compared to the untreated hemp biomass after PLE-EtOH/H_2O. Similarly, by 67-97% higher amount of other hexoses were obtained from hemp after EAE too. The content of disaccharide maltose was not detected in untreated sample at all, and only after treatment with enzyme was detected at the level of 0.52 g/100 g DW (**Table 20**). Generally, various factors, like plant growing conditions, harvest year, cultivar, and maturation can influence saccharide composition in fiber type hemp. In this study presence of Viscozyme® L enhance content of glucose by 94% as compared with sample without enzyme, these results are relatively similar to the yield reported in Moxley and Zhu (2008) studies. These authors showed that after 72 h of hemp stalks enzymatic hydrolysis with 60 international units (IU) of Novozyme 188 (β-glucosidase) at pH 4.8, glucose yield is 98% from glucan 42.37 g/100 g biomass was obtained glucose 41.85 g/100 g biomass [199]. These studies suggests that cellulolytic enzymes such as Viscozyme® L, Novozyme 188 or Genencor Spezyme CP could be applied for fiber type hemp cellulose hydrolysis, which enhance application of hemp stalks in fuel production and also could be used for bound valuable bioactive components isolation.

3.3. Characterization of cannabinoid and antioxidant fractions from *C. sativa 'Benico'* cultivar

3.3.1. SCE-CO_2, PLE and EAE extract yields under optimized conditions

The yields of SCE-CO_2, PLE and EAE extracts, isolated from mixture of seeds, blossoms and leaves of *C. sativa 'Benico'* cultivar under optimized extraction conditions (**Fig. 8**) are listed in **Table 21.** Values are expressed as g/100 DW of plant material prior each step of extraction and also recalculated to g/100 DW of starting plant material prior SCE-CO_2. In total, 51.69 g of extractable non-polar and polar constituents were isolated from 100 g of mixture of seeds, blossoms and leaves of *C. sativa 'Benico'* cultivar. The obtained results indicated that at the 1st step of biorefining process under the optimal SCE-CO_2 conditions (465 bar, 70°C, 120 min), *'Benico'* sample yielded 8.30 g/100 g DW of lipophilic fraction. At the 2nd and 3rd steps, 4.73 and 21.58 g of polar fraction was removed from 100 g of residue after SCE-CO_2 by means of PLE-Acetone (100°C, 45 min) and PLE-EtOH/H_2O (100°C, 45 min, EtOH/H_2O 4:1 v/v %), respectively, which is equal to 4.33 and 18.86 g/100 g DW of starting (non-extracted) *'Benico'* sample. Further PLE residue treatment with cellulolytic enzyme Viscozyme L additionally

released 20.20 g of hydrophilic constituents, as recalculated per 100 g of hemp prior SCE-CO_2. Therefore, high pressure extraction techniques contributed to the major portion (61%) of all extracted constituents and enzyme-assisted extraction – to the remaining 39%. Comparing these results with previously reported data (**Table 1**), SCE-CO_2 extract yield from hemp seeds varied from 16.8 to 44.2%. Mainly in all studies, reported by different authors, SCE-CO_2 extract yield is from 2 to 5.3 fold higher as compared to SCE-CO_2 extract yield from *'Benico'* sample, this difference could be explained by different anatomical parts and particle size.

Table 21. Extract yield of different extraction methods SCE-CO_2, PLE (Acetone and EtOH) and EAE-Viscozyme

Sample	Yield	
	*g/100 g DW**	*g/100 g DW s.m.***
SCE-CO_2	-	8.30 ± 0.01^{b}
PLE-Acetone	4.73 ± 0.13^{a}	4.33 ± 0.12^{a}
PLE-EtOH/H_2O	21.58 ± 0.59^{b}	18.86 ± 0.51^{c}
EAE-Viscozyme	29.48 ± 0.33^{c}	20.20 ± 0.23^{d}

*: yield g/100 g DW of sample prior extraction; **: yield g/100 g DW of starting material prior SCE-CO_2; Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$)

Comparing SCE-CO_2 and Soxhlet extraction, generally the latter technique leads to the higher oil yields [185, 200]. For example, Kostic and Jokovic (2013) reported that hemp seed (0.47 mm) oil yield was equal to 29.06 g/100 g DW when Soxhlet extraction with hexane was conducted at 70°C for 10 min. However, Soxhlet extraction efficiency may highly vary according to solvent type, extraction temperature, particle size and mixing intensity applied [201, 202, 203]. The main disadvantages of the conventional solvent extraction towards innovative high pressure fractionation techniques were discussed in Chapter 1.4. Previously, Porto and Decorti indicated no significant changes in fatty acids composition in both ways isolated oils [13], however oxidation stability of oil extracted by means of Soxhlet (0.84 (Eq αtoc/ml oil) was 2.2 fold lower as compared to SCE-CO_2 extract (1.87 (Eq αtoc/ml oil) [119]. Other study of Chen and He (2012) reports that ultrasonic bath treatment at 40 kHz for 30 min extraction with 100% and 75% of acetone and ethanol yields respectively 0.54 and 8.06% of extracts from defatted hemp seeds [8]. These data are in agreement with our study results, also indicating significantly higher (more that 4-fold) efficiency of ethanol towards polar constituent isolation from fiber-type hemp residue after SCE-CO_2, as compared to acetone.

3.3.2. Cannabinoid content in SCE-CO_2 extract and plant material under optimized conditions

CBD and CBDA amount in *'Benico'* SCE-CO_2 extract under optimized extraction conditions was already discussed in Section 3.2.1.2 and additionally compared with cannabinoid content in plant material before and after SCE-CO_2 in **Table 22**. Noticeable that in *'Benico'* sample CBDA content was significantly higher than CBD: before SCE-CO_2 extraction about 17 times, after

SCE-CO_2 extraction about 10 times. Obtained results indicates that the recovery of CBD and CBDA by SCE-CO_2 was almost 100 % and ~93%, respectively. Remaining CBD (0.003 mg/100 g DW) and CBDA (0.166 mg/100 g DW) amounts in hemp residue after SCE-CO_2 were negligible, as compared to the initial thereof concentrations in starting plant material. These results are in good agreement with data in Chapter 1.3 and additionally confirms the usefulness of SCE-CO_2 for target cannabinoid isolation from various anatomical parts of fiber-type hemps and their concentration in non-polar fractions (e.g. hemp seed oil).

Table 22. CBD and CBDA amount (g/100 g DW) in *'Benico'* variety hemp seeds, blooms and leafs mixture samples before and after SCE-CO_2

Compound	SCE-CO_2 extracts		Plant material	
			Before SCE-CO_2	After SCE-CO_2
	mg/g extract	*g/100 g DW*	*g/100 g DW*	*g/100 g DW*
CBD	24.72 ± 0.314^a	0.20 ± 0.003^a	0.14 ± 0.005^a	0.003 ± 0.000^a
CBDA	261.40 ± 2.243^b	2.17 ± 0.019^b	2.35 ± 0.041^b	0.166 ± 0.004^b

Different superscript letters within the same line indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$)

In Mechtler and Bailer (2004) study it was reported that hexane extract, obtained from *'Benico'* leaves, blossoms, small structural parts of the inflorescence and bracts (1.4 mm), contains 1.50 % of CBD [204], which is by 1.6 fold lower as compared to the results of our study (2.47% of CBD). Thus it can be deduced that SCE-CO_2 under suggested optimal conditions (465 bar, 70°C, 120 min) is more efficient than conventional non-polar solvent hexane based extraction for target cannabinoid fractionation.

3.3.3. Preliminary phytochemical analysis of SCE-CO_2, PLE and EAE extracts

The preliminary phytochemical composition of different *C. sativa 'Benico'* extracts (SCE-CO_2, PLE-Acetone, PLE-EtOH/H_2O), EAE-derived supernatant and corresponding water-soluble fraction from control sample (no enzyme added) was analysed by UPLC-QTOF-MS. The chromatograms of SCE-CO_2, PLE-Acetone, PLE-EtOH/H_2O, EAE-Viscozyme extracts are depicted in **Fig. 18**. Qualitative distribution of phytochemicals in EAE-Blank sample was identical to EAE-Viscozyme extract, only with some differences in total peak areas for each individual compound detected. Retention times, accurate masses, molecular ion [M-H] formulas and peak areas (in arbitrary units/g DW of starting plant material) of major SCE-CO_2, PLE-Acetone, PLE-EtOH/H_2O and EAE extract constituents are reported in **Table 23**. Concerning the variability of the data, the relative standard deviations of peak areas were < 5%.

As given in **Table 23**, peaks **1** (0.3 min, 387.1144 m/z, $C_{13}H_{23}O_{13}$), **2** (0.4 min, 439.0756 m/z, $C_{17}H_{11}N_8O_7$), **7** (2.2 min, 593.1512 m/z, formula not provided), **13** (4.3 min), **14** (4.3 min), **15** (4.5 min), **16** (4.5 min), **17** (5.6 min, 297.1541 m/z, $C_9H_{17}N_{10}O_2$), **18** (5.7 min, 367.2643 m/z,

$C_{25}H_{35}O_2$), **19** (5.8 min, 297.1524 m/z, formula not provided), **20** (5.9 min, 367.2643 m/z, $C_{25}H_{35}O_2$), **21** (6.0 min, 311.1685 m/z, $C_9H_{23}N_6O_6$), **22** (6.2 min, 311.1682 m/z, $C_{10}H_{19}N_{10}O_2$), **23** (6.3 min, 311.1677 m/z, formula not provided), **24** (6.6 min, 325.1841 m/z, $C_{10}H_{25}N_6O_6$), **28** (8.2 min, 357.2056 m/z, $C_{22}H_{29}O_4$), **29** (8.4 min, 367.2629 m/z, $C_{21}H_{31}N_6$), **30** (8.4 min, 367.2643 m/z, $C_{25}H_{35}O_2$), **25** (6.7 min), **31** (9.8 min), **32** (9.9 min), **33** (10.7 min), **34** (10.9 min)**,** comprising from 3 to $498 \cdot 10^9$ of arbitrary peak area units/g DW of starting plant material for different extracts were failed to be ascribed to the particular phytochemical at this step of the research. Looking at the preliminary tentative identifications for SCE-CO_2 extract (**Table 23**), peaks **26** (357.2071 *m/z*) and **27** (359.2228 *m/z*), eluting at 6.8 and 7.0 min, corresponded to molecular ion [M-H] formulas $C_{22}H_{29}O_4$ and $C_{22}H_{31}O_4$, and were ascribed to important fiber-type cannabinoids cannabidiolic acid and cannabigerolic acid. Cannabidiolic acid was detected in PLE-Acetone and PLE-EtOH/H_2O extracts, however having 6 and 36 fold lower peak areas, as compared to non-polar SCE-CO_2 fraction. It is interesting to note, that UPLC-QTOF-MS analysis of SCE-CO_2 extract indicated the presence of cannabigerolic acid, which is regarded as a precursor for CBD, which is characterized by anti-bacterial, anti-inflammatory and antioxidant properties [205]. This compound was found at approximately 5-fold lower levels, as compared to cannabidiolic acid in SCE-CO_2 extract and also mainly extracted from plant material via the lipophilic fraction removal (4-fold lower peak area in PLE-Acetone extract). These data adds to the above discussed results of this research, showing that the major portion of target cannabinoids is removed from plant material already at the 1st step of biorefining process by means of SCE-CO_2 under optimized conditions (465 bar, 70°C, 120 min). Similar trends were observed for not identified compounds **29** (367.2629 *m/z*, $C_{21}H_{31}N_6$) and **30** ($C_{25}H_{35}O_2$, 367.2643 *m/z*), both eluting at 8.4-min (6 fold lower peak areas for PLE-Acetone extract, and not detected in polar ethanol/water fraction). Most probably these peaks represent cannabinoid-type hemp phytochemicals too, however the identification at this step of the research it was not possible.

In PLE-EtOH/H_2O extract, compounds **6, 8, 9, 11** and **12** correlated to molecular ion formulas of $C_{27}H_{29}O_{16}$ (609.1461 *m/z*), $C_{27}H_{29}O_{14}$ (577.1563 *m/z*), $C_{21}H_{17}O_{12}$ (461.0725 *m/z*), $C_{21}H_{17}O_{11}$ (445.0776 *m/z*) and $C_{22}H_{19}O_{11}$ (459.0933 *m/z*) and were tentatively ascribed to flavonoids: rutin, apigenin derivative, luteolin derivative, baicalin derivative. The peak areas of these compounds varied from 6 to $46 \cdot 10^9$ of arbitrary peak area units/g DW of starting plant material and were generally detected in PLE-EtOH/H_2O fraction.

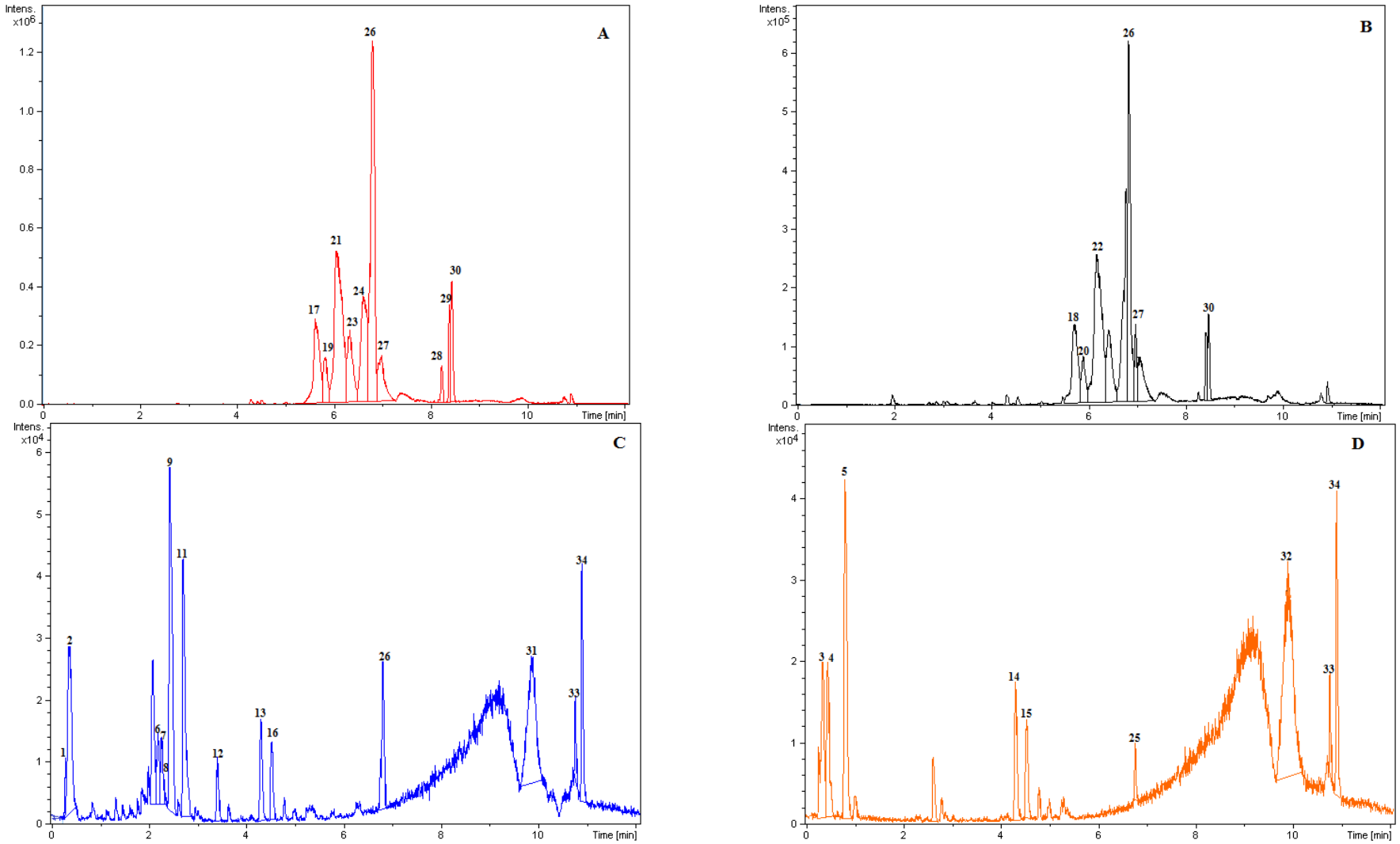


Fig. 18. UPLC/ESI-QTOF chromatographic profiles of phytochemicals in *C. sativa 'Benico'* SCE-CO_2 (A), PLE-Acetone (B), PLE-EtOH/H_2O (C), EAE-Viscozyme (D) extracts

Table 23. Characterization of individual compounds by means of UPLC/ESI-QTOF analysis in SCE-CO_2, PLE-Acetone, PLE-EtOH/H_2O extracts and water-soluble fraction and control sample after EAE from *C. sativa 'Benico'* cultivar

Peak No.	UPLC/ESI-Q-TOF			Peak area*, arbitrary units/g DW x10^9					Compound**
	RT (min)	*MS [M-H]$^-$ m/z*	*Formula[M-H]*	*SCE-CO_2*	*PLE-Acetone*	*PLE-EtOH*	*EAE-Viscozyme*	*EAE-Blank*	
1	0.3	387.1144	$C_{13}H_{23}O_{13}$	-n[d]	-	3	-	-	*Not identified*
2	0.4	439.0756	$C_{17}H_{11}N_8O_7$	-	-	33	-	-	*Not identified*
3	0.4	193.0354	$C_6H_9O_7$	-	-	-	19	2	Glucuronic acid
4	0.5	133.0142	$C_4H_5O_5$	-	-	-	16	11	Malic Acid
5	0.8	191.0197	$C_6H_7O_7$	-	-	-	33	24	Citric acid
6	2.1	609.1461	$C_{27}H_{29}O_{16}$	-	-	16	-	-	Rutin
7	2.2	593.1512	*Not provided*	-	-	16	-	-	*Not identified*
8	2.3	577.1563	$C_{27}H_{29}O_{14}$	-	-	7	-	-	Apigenin derivative
9	2.4	461.0725	$C_{21}H_{17}O_{12}$	-	-	46	-	-	Luteolin derivative
10	2.6	295.0459	$C_{13}H_{11}O_8$	-	-	-	5	4	Succinic acid
11	2.7	445.0776	$C_{21}H_{17}O_{11}$	-	-	32	-	-	Baicalin derivative
12	3.4	459.0933	$C_{22}H_{19}O_{11}$	-	-	6	-	-	Glucuronopyranoside derivative
13	4.3	*Not provided*	*Not provided*	-	-	10	-	-	*Not identified*
14	4.3	*Not provided*	*Not provided*	-	-	-	10	6	*Not identified*
15	4.5	*Not provided*	*Not provided*	-	-	-	9	4	*Not identified*
16	4.5	*Not provided*	*Not provided*	-	-	8	-	-	*Not identified*
17	5.6	297.1541	$C_9H_{17}N_{10}O_2$	204	-	-	-	-	*Not identified*
18	5.7	367.2643	$C_{25}H_{35}O_2$	-	50	-	-	-	*Not identified*
19	5.8	297.1524	*Not provided*	77	-	-	-	-	*Not identified*
20	5.9	367.2643	$C_{25}H_{35}O_2$	-	21	-	-	-	*Not identified*
21	6.0	311.1685	$C_9H_{23}N_6O_6$	498	-	-	-	-	*Not identified*
22	6.2	311.1682	$C_{10}H_{19}N_{10}O_2$	-	123	-	-	-	*Not identified*
23	6.3	311.1677	*Not provided*	163	-	-	-	-	*Not identified*
24	6.6	325.1841	$C_{10}H_{25}N_6O_6$	265	-	-	-	-	*Not identified*
25	6.7	*Not provided*	*Not provided*	-	-	-	3	1	*Not identified*
26	6.8	357.2067	$C_{22}H_{29}O_4$	619	103	17	-	-	Cannabidiolic acid
27	7.0	359.2228	$C_{22}H_{31}O_4$	121	31	-	-	-	Cannabigerolic acid
28	8.2	357.2056	$C_{22}H_{29}O_4$	31	-	-	-	-	Cannabidiolic acid
29	8.4	367.2629	$C_{21}H_{31}N_6$	60	-	-	-	-	*Not identified*
30	8.4	367.2643	$C_{25}H_{35}O_2$	107	18	-	-	-	*Not identified*
31	9.8	*Not provided*	*Not provided*	-	-	45	-	-	*Not identified*
32	9.9	*Not provided*	*Not provided*	-	-	-	68	20	*Not identified*
33	10.7	*Not provided*	*Not provided*	-	-	4	5	2	*Not identified*
34	10.9	*Not provided*	*Not provided*	-	-	18	19	8	*Not identified*

*: recalculated as arbitrary units/g DW of starting plant material x10^9 taking into account yields (8.30 for SCE-CO_2, 4.33 DW for PLE-Acetone, 18.86 for PLE-EtOH/H_2O, 20.2 for AEA-Viscozyme, 9.4 for AEA-Blank g/100 g DW of starting material), sample concentration (1 mg/mL) and injection volume (1 μL); **: tentatively identified

The composition of flavonoids are similar to reported by Sanchez and Verpoorte (2008) [97] and Srivastava and Chauhan (2012) [98], the vast majority of flavonoids are extracted with ethanol or other polar solvents, e.g. water. In aqueous extracts of *C. sativa* leaves and blossoms amount of flavonoids was found at 0.7 and 2.7 mg quercetin equivalent/g extract. As reported by Sanchez and Verpoorte, in methanol and chloroform extracts from *C. sativa Kompolti'* and *'Fasamo'* cultivars seeds and leaves following flavonoids were detected: orientin (0.004-0.6 mg/100 g DW), vitexin (0.29-0.13 mg/100 g DW), isovitexin (0.04-0.09 mg/100 g DW), quercetin (0.28-0.27 mg/100 g DW), luteolin (0.49-0.48 mg/100 g DW), kaempferol (0.005-0.26 mg/100 g DW) and apigenin (0.61-1.2 mg/100 g DW) [97].

In EAE-Viscozyme and EAE-Blank extracts matching molecular ion formulas of $C_6H_9O_7$ (193.0354 *m/z*), $C_4H_5O_5$ (133.0142 *m/z*) and $C_6H_7O_7$ (191.0197 *m/z*) for peaks **3**, **4** and **5** tentatively suggested the presence of glucuronic, malic and citric acids. Additionally was observed that the content of those acids and other unidentified constituents in EAE-Blank extract was approximately form 1.3 to 9.5 folds lower, as compared to EAE-Viscozyme extract. These results additionally confirms the efficiency of enzymatic hydrolysis to enhance the extraction of water-soluble hemp constituents from residue after PLE-EtOH/H_2O [206].

3.3.4. In vitro antioxidant activity assessment of Benico extracts and plant material

In vitro antioxidant activity of SCE-CO_2, PLE and EAE extracts and plant material was evaluated as total phenolic content (mg GAE/g), ferric reducing power (mg TE/g), DPPH$^{\bullet}$ (mg TE/g), ABTS$^{\bullet+}$ (mg TE/g) and ORAC (mg TE/g) radical scavenging capacity and also recalculated per gram of starting material prior SCE-CO_2 (**Table 24**) Since the solid residues after various steps bio-refining process may still retain antioxidatively active constituents, antioxidant potential of water-non soluble fractions was assessed by QUENCHER approach [13] and expressed as mg GAE or TE/g of starting material prior SCE-CO_2. In all assays no significant differences in TPC and TEAC values for soluble and insoluble EAE-Viscozyme and EAE-Blank fractions were obtained, therefore the data of control sample (no enzyme added) were not listed in **Table 24** and will not be further discussed in this study.

As given in **Table 24**, TPC content ranged from 3.68 to 124.70 mg GAE per gram of extract. Taking into account the yields of extracts (**Table 21**), these values amounted 0.86 to 23.52 mg GAE/g DW of starting material prior SCE-CO_2. In total, all extracts contributed to 38.00 mg GAE per gram of starting material, with the share decreasing as follows: PLE-EtOH/H_2O > SCE-CO_2 > PLE-Acetone > EAE-Viscozyme. The ferric reducing power of tested extracts varied from 17.40 to 457.08 mg TE per gram of extract, corresponding to 2.24-86.20 mg TE/g DW of starting material prior SCE-CO_2. Similarly to Folin-Ciocalteu's assay, the highest share to the total activity (110.41 mg TE/g DW prior SCE-CO_2) was found for *Benico* polar ethanol/water extract, followed by the 5.6, 15.8 and 24.5 fold less active PLE-acetone, SCE-CO_2 and EAE-Viscozyme extracts. Looking at the radical scavenging capacity, $TEAC_{DPPH}$ values of 2.99-72.51mg TE/g extract, $TEAC_{ABTS}$ values of 40.89-1059.8 mg TE/g extract and $TEAC_{ORAC}$ values of 83.74-1088.08 mg TE/g extract were obtained. These results, expressed per gram DW of unextracted sample, were in the range of 0.59-11.13 mg TE for DPPH$^{\bullet}$, 49.97 to 226.96 mg TE for ABTS$^{\bullet+}$ and 7.87-205.21 mg TE for ORAC. In total, all extracts contributed to 16.34 (DPPH$^{\bullet}$), 302.57 (ABTS$^{\bullet+}$) and 264.21 (ORAC) mg of TE per gram of starting material and the sequence of individual activities was:

PLE-EtOH > SCE-CO_2 (2.2-5.6 fold lower) > PLE-Acetone (3.1-16.7 fold lower) > EAE-Viscozyme (on average, 18.5 fold lower). The highest activity of PLE-EtOH/H_2O extract is in agrrement by study of Chen and He in 2012. Authors assessed radical scavenging properties of acetone, ethanol and methanol extracts, isolated from *C. sativa 'Bama'* cultivar, by means of DPPH IC_{50} and ABTS IC_{50} and found ethanol extract to be the most active too: 1) DPPH: Ethanol (1.89-2.56 mg/ml) > Acetone (1.33 to 2.07 mg/ml) > Methanol (1.01 to 1.91 mg/ml); 2) ABTS: Ethanol (0.177-0.197 mg/ml) > Acetone (0.108-0.128 mg/ml) > Methanol (0.085-0.485 mg/ml) [8].

Table 24. Total phenolic content (TPC), ferric reducing antioxidant power (FRAP), DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and ORAC scavenging properties of SCE-CO_2, PLE and EAE extracts from *C. sativa 'Benico'* cultivar and plant material before and after various steps of extraction

Sample	Extract			Plant material	
				Before extraction	*After extraction*
	mg /g extract	*mg /g DW**	*mg /g DW s.m***	*mg /g DW s.m***	*mg /g DW s.m***
			TPC, mg GAE/g:		
SCE-CO_2	107.41±0.62^{b}	8.91±0.05^{b}	8.17 ± 0.05^{b}	35.49±1.37^{d}	28.91 ± 0.54^{d}
PLE-Acetone	115.84 ±2.19^{b}	5.48 ± 0.10^{b}	5.02 ± 0.09^{b}	28.91 ± 0.54^{c}	11.15 ± 0.47^{c}
PLE-EtOH/H_2O	124.70 ± 2.47^{c}	26.90 ± 0.53^{c}	23.52 ± 0.47^{c}	11.15 ± 0.47^{b}	4.18 ± 0.32^{b}
EAE-Viscozyme	6.38 ± 0.21^{a}	1.88 ± 0.06^{a}	1.29 ± 0.04^{a}	4.18 ± 0.32^{a}	2.95± 0.11^{a}
			$TEAC_{FRAP}$, mg TE/g:		
SCE-CO_2	71.49±0.80^{b}	5.93±0.07^{a}	5.44 ± 0.06^{a}	80.12 ± 0.94^{d}	68.39 ± 2.87^{c}
PLE-Acetone	352.18 ± 15.79^{c}	16.66 ± 0.61^{b}	15.25 ± 0.56^{b}	68.39 ± 2.87^{c}	53.84 ± 2.86^{b}
PLE-EtOH/H_2O	457.08 ± 10.86^{d}	98.64 ± 3.16^{c}	86.20 ± 2.77^{c}	53.84 ± 2.86^{b}	10.69 ± 0.38^{a}
EAE-Viscozyme	17.40 ± 1.59^{a}	5.13 ± 0.39^{a}	3.52 ± 0.27^{a}	10.69 ± 0.38^{a}	8.27 ± 0.38^{a}
			$TEAC_{DPPH}$, mg TE/g:		
SCE-CO_2	72.51±1.39^{e}	6.02±0.12^{c}	2.52 ± 0.11^{b}	41.88 ± 0.96^{d}	38.11 ± 0.48^{d}
PLE-Acetone	48.31 ± 1.62^{c}	2.29 ± 0.08^{b}	2.09 ± 0.07^{b}	38.11 ± 0.48^{c}	29.21 ± 1.33^{c}
PLE-EtOH/H_2O	58.99 ± 2.00^{d}	12.73 ± 0.39^{d}	11.13 ± 0.34^{d}	29.21 ± 1.33^{b}	4.28 ± 0.23^{b}
EAE-Viscozyme	2.99 ± 0.08^{a}	0.88 ± 0.02^{a}	0.60 ± 0.02^{a}	4.28 ± 0.23^{a}	1.02 ± 0.05^{a}
			$TEAC_{ABTS}$, mg TE/g:		
SCE-CO_2	1024.7 ± 13.38^{c}	85.05 ± 1.11^{c}	77.99 ± 1.01^{c}	189.59 ± 9.26^{c}	199.88 ± 10.45^{c}
PLE-Acetone	1059.8 ± 88.84^{c}	50.13 ± 3.45^{b}	45.89 ± 3.16^{b}	199.88±10.45^{c}	110.73 ± 5.38^{b}
PLE-EtOH/H_2O	898.31 ± 67.15^{b}	193.81±13.96^{d}	169.42±12.20^{d}	110.73 ± 5.38^{b}	24.84 ± 0.37^{a}
EAE-Viscozyme	45.91 ± 10.25^{a}	13.54 ± 2.64^{a}	9.27 ± 1.81^{a}	24.84 ± 0.37^{a}	20.12 ± 0.39^{a}
			$TEAC_{ORAC}$, mg TE/g		
SCE-CO_2	469.55 ± 21.91^{c}	38.93 ± 1.81^{a}	35.68 ± 1.65^{b}	174.79 ± 21.36^{bc}	146.31 ± 13.83^{b}
PLE-Acetone	282.41 ± 18.93^{b}	13.36 ± 0.90^{a}	12.23 ± 0.82^{a}	146.31 ± 13.83^{b}	183.47 ± 21.29^{c}
PLE-EtOH/H_2O	1088.08±109.22^{d}	234.81±23.57^{b}	205.21±20.60^{c}	183.47 ± 21.29^{c}	21.24 ± 1.91^{a}
EAE-Viscozyme	54.30 ± 3.36^{a}	16.01 ± 0.99^{a}	10.97 ± 0.68^{a}	21.24 ± 1.91^{a}	2.36 ± 0.13^{a}

*- mg GAE or TE/g plant material prior each step of extraction; **- mg GAE or TE/g DW of plant material prior SCE-CO_2; Different superscript letters within the same column for individual assays indicate significant differences one way ANOVA and Tukey's test, $p < 0.05$)

Looking at the results of insoluble fractions, in most cases TPC and TEAC values of starting plant material (prior extractions) were the highest, amounting 35.5 mg GAE and 41.9-189.6 mg TE per gram DW. The combined SCE-CO_2, PLE and EAE reduced initial total phenolic content of *C. sativa* '*Benico*' cultivar sample by 92%, FRAP activity by 90%, and DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and ORAC radical scavenging capacities by 98%, 89% and 99%, respectively. At different steps of biorefining process (**Fig. 8**), up to 19% of the initial plant material activity was reduced after

lipophilic fraction isolation by means of SCE-CO_2 with further 18-50% and 19-71% decrease due to the polar compounds removal with acetone and ethanol/water mixture, respectively. EAE additionally extracted the remaining 2-12% of water soluble constituents with *in vitro* reducing power and radical scavenging properties. It can be concluded that the suggested biorefining protocol under optimized conditions (**Fig. 8**) is efficient to remove the major portion of non-polar and polar constituents with *in vitro* reducing power and radical scavenging properties from *C. sativa* '*Benico*' cultivar.

3.4. Evaluation of CBD and CBDA preparations toxicity on Caco-2 cells

For the purposes of this research CBD and CBDA preparations toxicity is evaluated, further non-toxic concentrations of CBD and CBDA preparations are used for proteins expression (by Western Blot) and proteins immunolocalization (by immunofluorescence) evaluation on CaCo-2 cells. For the toxicity evaluation, two preparations containing 100% of CBD and 27% of CBDA, respectively, were tested employing TEER (Transepithelial Electrical Resistance) and PRP (Phenol Red Passage) assays. TEER measurement is one of the easiest, trustworthy and essential method to monitor and evaluate the growth of epithelial tissue cultures *in vitro*. Decrease in TEER values observed during the particular exposure time refers to the increased cell damage level and, in consequence, higher epithelial monolayer permeability [72, 207]. Effect of target plant bioactives on cell membrane permeability also can be verified by assessing the paracellular flux of the phenol red dye. Increased cell permeability can be assessed by higher phenol red concentration in the medium after diffusion through cells monolayer [176, 208, 209]. Currently, various cell lines and their co-cultures are utilized for *in vitro* toxicity and biological response assessment models, with human colorectal adenocarcinoma-derived CaCo-2 cells being most widely applied to study nutrient and drug transport. These cells can be maintained easily in cell culture for many weeks, are capable of establishing tight junctions in culture and provide the main routes of target substance transport via four main mechanisms: 1) the paracellular route (forming tight junction complexes), 2) the passive transcellular route (through the bodies of epithelial cells), 3) the active carrier-mediated paracellular (through cells), and 4) transcytosis [210] Additionally, results obtained in the presence of target substance are commonly compared with those of known membrane permeability and cell-damage inducer (e.g., AAPH). For the purposes of this study, the effect of CBD (100%) and CBDA (27%) preparations were tested at the concentration range of 1-10 μM, in the absence or presence of free radical generator AAPH (**Fig. 19**). As expected, the highest membrane permeability and damage level were obtained for AAPH-treated cells (without

cannabinoid preparation added), showing 57 % reduction in the initial TEER value after 3.5 hours of treatment. On the contrary, cells treated with CBD and CBDA preparations demonstrate only 15-22% and 6-17% decrease in the TEER values, measured at the onset of the experiment, respectively, with the lowest TEER values obtained only at the highest cannabinoid concentrations tested (10 μM). The membrane permeability of cells treated with 1-5 μM preparations remains practically intact (6-17%), especially in the case of CBDA, and correlates well with observer TEER decrease level in untreated sample (~6%). Additional experiments were conducted to assess the effect of both preparations on the overall toxicity of AAPH. As depicted in **Fig. 19** up to 47% decrease in TEER values was obtained for CBD or CBDA (1-10 μM) + AAPH treated samples, which is lower as compared to AAPH treatment alone. Therefore, the presence cannabinoids in AAPH-treated samples increases cell membrane resistance for AAPH-induced damages.

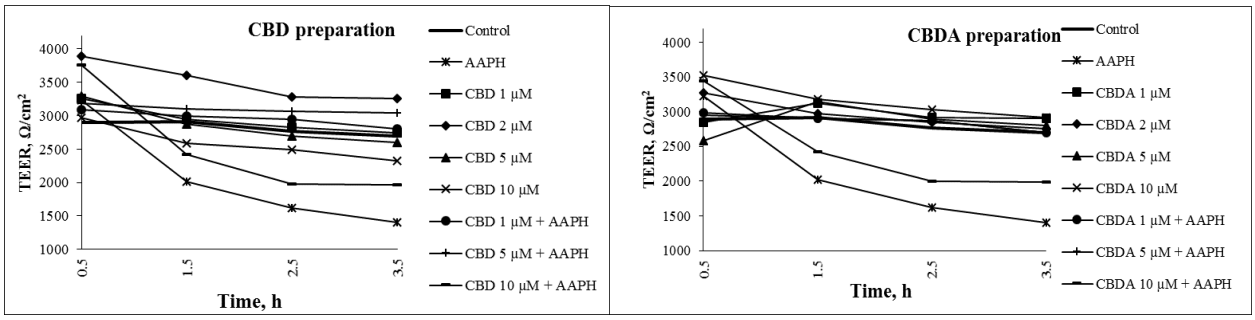


Fig. 19. Evaluation of CBD and CBDA preparations toxicity on Caco-2 cells by TEER method

Similar effects of tested cannabinoid preparations were observed comparing AAPH and cannabinoid-treated cell membrane permeability in PRP assay. As given in **Fig. 20**, CBD and CBDA-treated cells demonstrate similar *Papp* values [0.39-0.65 and 0.17-0.58 $(cm \times s^{-1}) \times 10^{-6}$, respectively] to control sample [0.48 $(cm \times s^{-1}) \times 10^{-6}$], which in both cases is significantly lower as compared to AAPH-derived ones [1.66 $(cm \times s^{-1}) \times 10^{-6}$].

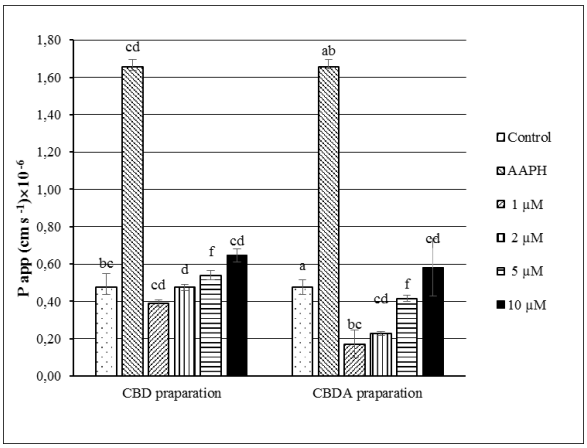


Fig. 20. Passage of phenol red across Caco-2 cells grown

Therefore, results of both assays indicate no or very low toxicity level of both cannabinoid preparations on CaCo-2 cells at the tested concentration range. Similar observations were previously reported by Alhamoruni and Lee (2010), showing that the EDTA-induced increase permeability of Caco-2 cells is completely inhibited by 10 μM CBD or other cannabinoids. This suggests that CBD could be used to protect cells against damages and inhibit increased gut permeability [211].

3.5. Effect of CBD and CBDA preparations on Caco-2 cells monolayer integrity

3.5.1. Expression of NF-κB by Western blotting assay

Western blotting assay is often used for separation and identification of various proteins (from the mixture of proteins). Separation (through gel electrophoresis) is based on molecular weight and type. Inflammation is a normal physiological response to infections or injuries, initiating pathogen killing and tissue repair processes, which are necessary to restore damaged sites [212]. Protection mechanism is based on the instant identification of molecular microbes models by receptors, expressed in the cell membrane and nucleotide-binding. In response to the attack of bacteria, the signals are directed to transcription factors NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which initiate genes, responsible for the synthesis of pro-inflammatory proteins, transcription [212]. NF-κB is a complex of proteins that control transcription of DNA, cytokine production and cell survival [213, 214]. This complex is found in all animal cell types and is involved in cellular responses to stress, free radicals, ultraviolet irradiation, bacterial or viral antigens stimulation and regulates the immune response to infection [215, 216, 217]. Abnormal and increased regulation of NF-κB informs about cancer, inflammatory and autoimmune diseases, viral infection, septic shock, bowel disease, arthritis, sepsis, gastritis, asthma, atherosclerosis and et cetera [218, 219, 220, 221, 222, 223, 224]. One of the most common techniques used to identify specific proteins from a mixture of proteins that are extracted from cells is Western blotting (WB). Utilizing this technique it is very important to: (1) separate cells by size, (2) transfer to a membrane and mark target protein with correct primary and secondary antibodies [225]. As presented in **Fig. 21**, treatment of CaCo-2cells with AAPH enhanced the expression of NF-κB from 39% to 65%, comparing with the control sample. The expression of NF-κB also was studied in the CaCo-2 cells treated with 1 μM and 5 μM cannabinoid preparations.

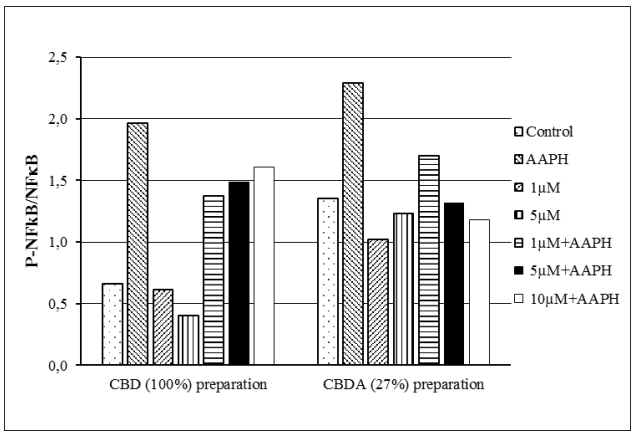


Fig. 21. The expression of NF-κB in CaCo-2 cells treated with CBD (100%) and CBDA (27%) preparations

Low NF-κB expression (equal to or lower than of control sample) obtained indicates that CBD and CBDA does not induce inflammatory status in cells. Cell exposure to combined cannabinoid preparation/AAPH treatment increased NF-κB expression by 17-56%, which is approximately 20% lower than cells affected by AAPH. The preliminary results on the expression of NF-κB, reveal that treatment with CBD partially protect cells from inflammatory status induced by treatment with AAPH, while cells treated with 5 and 10 μM CBDA and AAPH show a lower expression of NF-κB and demonstrate that CBDA is more able to inhibit the NF-κB-inflammatory pathway induced by oxidative stress.

3.5.2. *Expression of β-catenin and occludin by Western blotting assay*

Intestinal epithelium constitutes a barrier, involving two type of junctions (tight and adherens), which are intercellular complexes of cells that provide a continuous line around the apical region of the cells [226]. Cell junctions occur at points of cell-cell and cell-matrix collide in tissues, sustain the integrity of tissues, participate in signal transfer and determined by protein complex, involved in a signal transduction [227, 228]. The tight junction is a site where two cells come very close together (the outer layers of the cells membranes appear to be fused) and act as a barrier blocking substances diffusion between two interacting cells [229, 230, 231]. Occludin (integral plasma-membrane protein) is one of the major protein in a tight junction, securing its assembly, stability and barrier function [232] and showing morphological stability in several epithelial tissues [233]. Adherens junctions, found at sites of cell interaction, and central contacts, intermediating cells with the extracellular matrix, links cells together or to the extracellular matrix and transport signals into and out of the cell [234, 235, 236]. In adherens junctions, β-catenin is one of the major transmembrane adhesion molecules, binding to E-cadherin at cellular junctions,

which is fundamental for appropriate adherens junctions organization. Additionally, β-catenin acts as regulator and coordinator of cells adhesion and genes transcription, and as transporter of intracellular signals in Western blot assay [237, 238, 239, 240]. Over-expression of β-catenin and occludin is associated with many cancers, various forms of heart diseases, including dilated cardiomyopathy and other illness [241], higher expression of these proteins indicates damages in cells.

For the purposes of this research, the expression of two major junction proteins β-catenin and occludin in the presence or absence of CBD (100%) and CBDA (27%) preparations was tested utilizing CaCo-2 cells. The obtained results are depicted in **Fig. 22.** As expected, treatment of cell culture with control compound AAPH enhanced the expression of β-catenin and occludin proteins (up to 55% on average) as compared to those of untreated cells. This AAPH-induced protein expression was reduced up to 2.5 fold due to the addition of CBD and especially CBDA to AAPH. In addition, treatment of CaCo-2 cells only with CBDA did not provoke the alterations of β-catenin and occludin proteins expression. Cells treated with CBD exerted higher β-catenin and occludin proteins expression than those treated with CBDA, which additionally suggest stronger protective effect of CBDA than of CDB against cells damages, induced by, for example, oxidative stress.

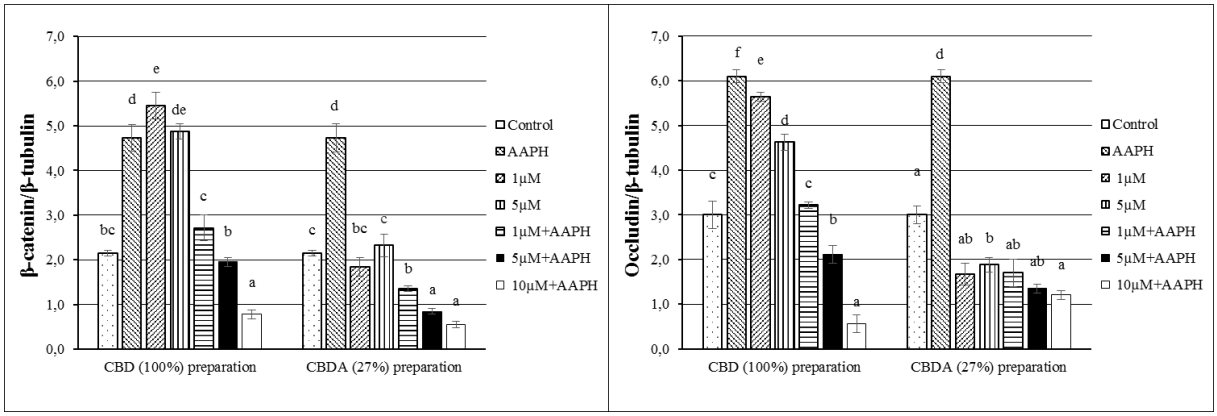


Fig. 22. The expression of β-catenin and occludin proteins in CaCo-2 cells treated with CBD (100%) and CBDA (27%) preparations

Summarizing, the treatment of CBD and CBDA preparations has positive effect and inhibit damages induced by AAPH in cells. These results are in agreement with studies reported by Borrelli and Aviello (2009), showing that CBD has positive effect against oxidative stress in human colon adenocarcinoma. At the concentration range of 1-10 μM this cannabinoid reduce colon injury, reactive oxygen production and lipid peroxidation in CaCo-2 cells. As concluded by these authors, CBD is likely safe compound and prevents experimental colitis in mice [242]. More recently Harvey and Sia (2014) conducted experiments on CaCo-2 cells, affected with IL-17A

(interleukin17A, with the purpose to evoke mucosal inflammation) and 10 μM CBD. The obtained results showed that cannabinoid treatment did not altered IL-17A expression, which was explained by CBD potential protection towards human colon from damages [73]. It is known that CBD can inhibit IL-17A expression and control inflammation, gut permeability of epithelial paracellular and mucosal damages [243, 244, 245, 246, 247]. In addition to these observations, recent results of Rock and Parker (2013) study outline the inhibiting capacity of CBDA and receptor antagonist (ondansetron) towards highly aggressive human breast cancer cells migration: together those compounds play important role in cancer treatment, reduce the symptoms of nausea and vomiting and reduce cancer cells migration (an important factor in cancer metastasis) [42].

3.5.3. *Expression of CB1 and CB2 receptors antibodies by Western blotting assay*

As reported in **Fig. 23** treatment with AAPH enhanced the expression of CB1 and CB2 receptors antibodies up to 20% in CaCo-2 cells as compared to control, higher expression indicates damages in cells. Expression of CB1 and CB2 receptors antibodies in samples treated with combined CBD/AAPH and CBDA/AAPH was by 0-33% higher than of control sample. The expression of CB1 and CB2 receptors antibodies was slightly increased in cells treated with CBDA (**Figure 5**). Treatment with all concentrations of CBD induced an increase of CB1 receptor antibody expression, while CB2 receptor antibody expression was not affected. However obtained results are not precise and clear, for more accuracy additional studies are required.

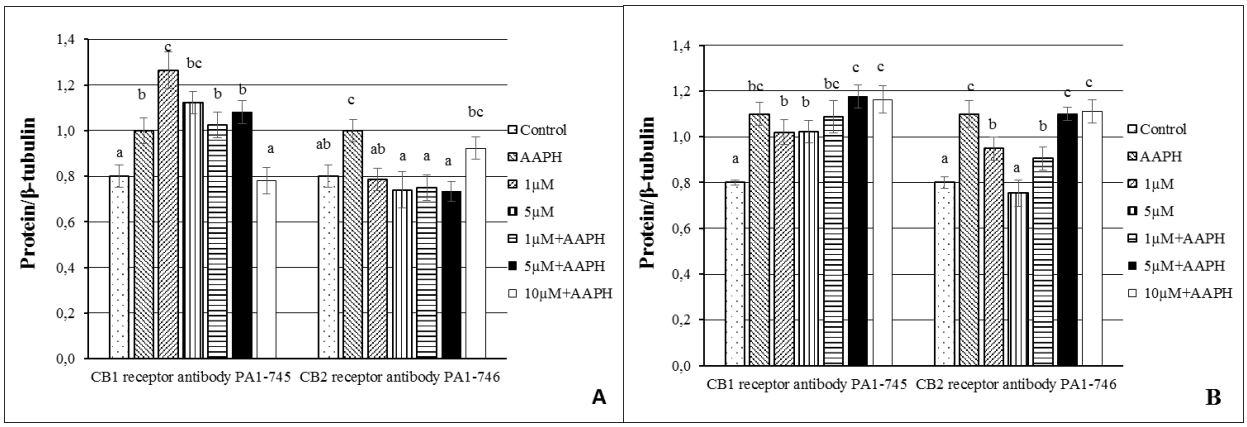


Fig. 23. The expression of cannabinoid CB1 and CB2 receptors antibodies in CaCo-2 cells treated with CBD (100%, A) and CBDA (27%, B) preparations

Previously several research groups showed that both CBD and CBDA may modulate the endocannabinoid system through an indirect action on cannabinoid receptors [83], [84], [248], [66], [249]. Ligresti and Moriello (2006) showed that CBD and especially CBD-enriched extract neutralizes tumour cells growth (particularly for a human breast cancer cell line) and have a

promising application in cancer medicine priducing. These authors suggested that bioactivity of CBD works through direct or indirect activation of CB2 receptors [250]. More recently, Alhamoruni and Wright (2012) reported that CBD (10μM) is acting via CB1 receptors and is able to change permeability of cells, phytocannabinoids could be applied for increasing reversing in damaged intestinal permeability [211, 251]. Generally, it could be concluded that cannabinoids action mechanism via CB1 and CB2 receptors is still not clear and the exact model of cannabinoids action with receptors is not established yet.

3.5.4. *Localization f ZO-1 protein and occludin by immunofluorescence*

In addition to Western blotting assay, immunofluorescence (technique for specific target antigens' detection) could be applied to monitor the expression of specific junction proteins in the presence of bioactive constituents. Conducting immunofluorescence measurements, antibodies are labelled with fluorescent dyes, such as fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC), and antibody-antigen complexes are detected by microscope [252, 253, 254, 255, 256]. For the purposes of this research, immunolocalization of junction protein ZO-1 and distribution of occludin in cell membranes [173] was performed to determine whether CBD, CBDA and, in comparison, AAPH, at various concentrations affect the correct distribution of proteins. ZO-1 belongs to the family of tight junction proteins that acts as cross-linkers [257]. ZO-1 involved in the coordination and grouping of protein complexes to the cell membrane and in the creation of specialized area inside the membrane [258]. ZO-1 interacts with the other transmembrane protein occludin, a second tight junction-specific protein (*Section 1.2.2*) [259]. Generally, protein occludin is more sensitive to structural damages than ZO-1. Its dephosphorisation coincides with the onset of inflammation and leads to further moves from periphery to cytoplasm when this process continues [260]. Results in **Fig. 24 A** show intact immunolocalization of TJ proteins ZO-1 and occludin in untreated (control) cells (correct localization of proteins, comparing thin membranes network). Vice versa, clear delocalization of TJ proteins ZO-1 (diffused ZO-1 inside the cells) and occludin (discontinuous cell line) could be observed due to AAPH presence in the CaCo-2 cells (**Fig. 24 B**). Treatment with 5 μM CBD (**Fig. 24 C)** results in correct distribution of ZO-1 and occludin on the periphery of cells, while the highest concentration of this cannabinoid tested (10μM) already shows reduced presence of ZO-1 and occludin in plasma membrane, diffusion of these proteins inside the cells and some continuous line disappearing (**Fig. 24 D**).

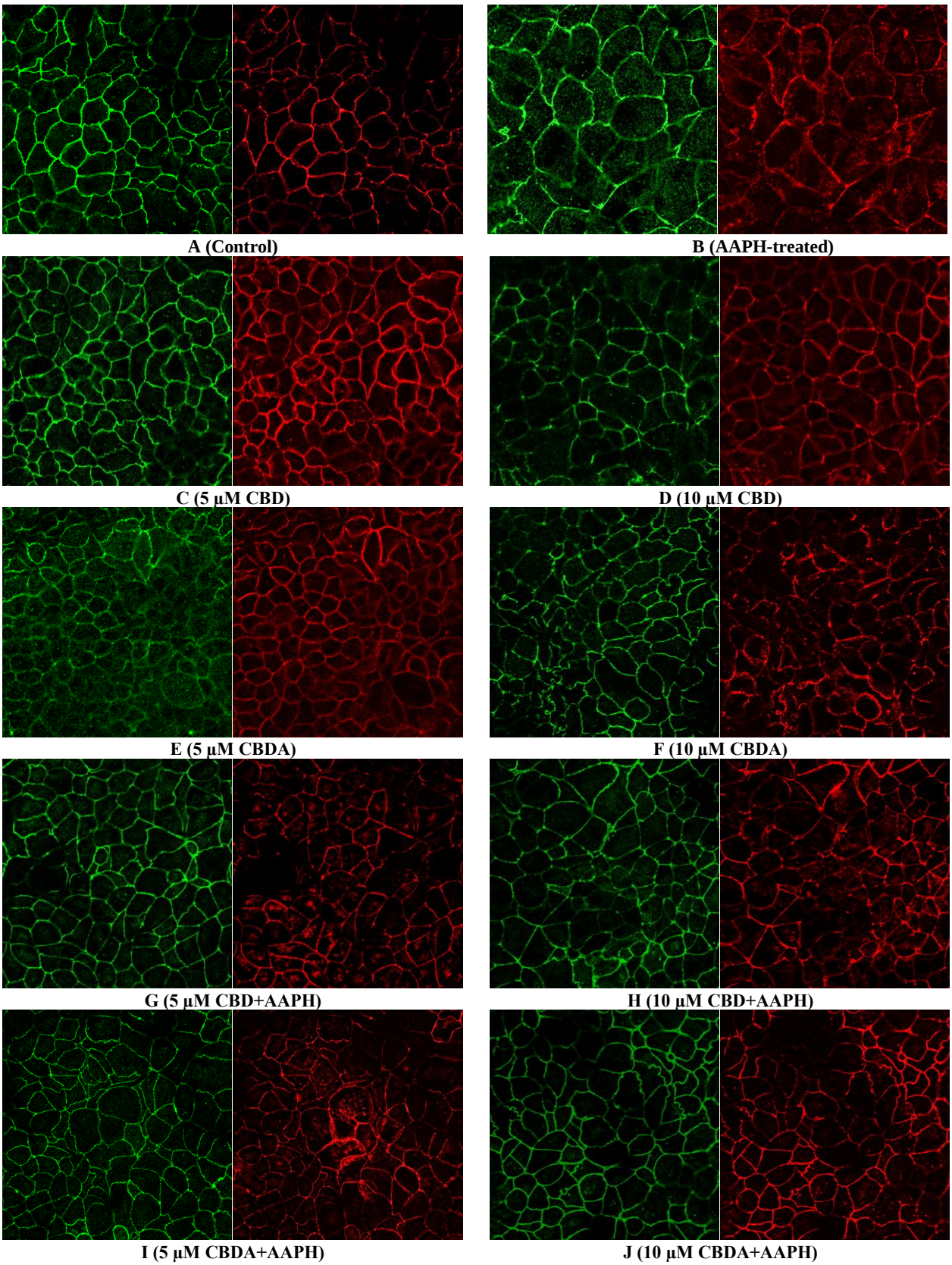


Fig. 24. Immunofluorescence of tight junction protein ZO-1(green) and occludin (red) localization in control (**A**), AAPH (**B**) and cannabinoid preparation [CBD (100%) and CBDA (27%), **C-J**] treated Caco-2 cells

On the contrary to CBD results, delocalization of tight junction proteins was observed for Caco-2 cells exposed to 5 μM CBDA preparation: occludin and, to a greater extent, ZO-1 (less continuous network) are diffused inside the cells and membrane lines are discontinuous (**Fig. 24 E**). With 10μM CBDA treatment (**Fig. 24 F**), both ZO-1 and occludin are distributed inside the cells, however ZO-1 is localized in more continuous network as compared to occludin (the linear structure of protein is discontinuous and protein is diffused inside the cells). The effects observed for combined cannabinoid/AAPH treatments are the following**.** 5μM of CBD: ZO-1 is localized in the cell membrane, occludin is diffused inside the cells (**Fig. 24 G**). In cells treated with 10μM CBD: both proteins are diffused inside the cells, however protein ZO-1 contains more linear structure as compared to occludin, where the vast majority of protein is diffused inside the membrane (**Fig. 24 H**). 5μM of CBDA: delocalization of ZO-1 and occludin proteins, however higher diffusion level of occludin inside the cells as compared to ZO-1(**Fig. 24 I**). 10μM of CBDA: only small content of proteins are diffused inside the cells, ZO-1 is more localized on the periphery of membrane as compared to occludin (**Fig. 24 J)**. The obtained data suggest that the higher concentration of CBD, and particularly of CBDA, is able to protect the epithelial barrier from inflammation induced by oxidative stress, as reduced activation of NF-κB inflammatory pathway shows. In addition, AAPH-induced damages of cells could be partially reduced by addition of cannabinoid preparations. Localization and expression of tight junction proteins indicates that the treatment of cells with 10 μM CBDA preparation was able to partially protect cells against damages induced by AAPH, however ocalization and expression of occludin indicates damages of cells membranes. Therefore, cannabinoids have the ability to modulate paracellular permeability, depending on dose of treatment and application type [211]. Nevertheless, more studies are needed in order to clarify if this effect might have a biological relevance in humans.

IV. CONCLUSIONS

1. The determined optimal SCE-CO_2 conditions for unspecified *C.sativa* variety were 400 bar, 40°C and 80 min and yielded 21.61 g/100 g of SCE-CO_2 extract, containing 0.616 g CBD/100 g DW and 0.086 g CBDA/100 g DW. Under these conditions 0.18-21.93 g/100 g DW of SCE-CO_2 extract from different *C.sativa* cultivars were obtained.
2. Under optimal conditions, SCE-CO_2 extracts from different *C.sativa* cultivars contained 0.001-0.211 and 0.001-1.923 g of CBD and CBDA per 100 g DW, respectively. Nearly all CBD and the major part of CBDA (up to 80%) was isolated from raw material and concentrated in lipophilic fraction. Antioxidant capacity of different *C.sativa* samples varied according to the cultival, vegetation period and anatomical part tested (1.65-6.97 mg GAE/g DW and 0.29-111.87mg TE/g DW). *C.sativa* cultivar *'Benico'* contained the highest amount of CBDA, showed the highest efficiency of SCE-CO_2 to concentrate CBD (99%) and CBDA (88%) in lipophilic fraction, and distinguished by the highest antioxidant capacity for SCE-CO_2 extracts and plant material before and after extraction.
3. 4-step biorefining protocol was developed for *C. sativa* cultivar *'Benico'.* Under optimized SCE-CO_2 conditions (465 bar, 70°C and 120 min), extract yield was 8.30 g/100 g DW, CBD – 0.205 g/100 g DW, CBDA – 2.169 g/100 g DW. The recovery of CBD and CBDA by SCE-CO_2 was ~ 100 % and 93%, respectively. Optimized PLE yielded 4.33 g/100 g DW of acetone extract (45 min, 100°C) and 18.86 g/100 g DW of ethanol/water extract (45 min, 100°C, EtOH/H_2O, 1/4 v/v %.). Additional 20.20 g/100 g DW of water-soluble constituens were removed via EAE-Viscozyme.
4. Antioxidant capacity of non-polar and polar fractions of *C. sativa* cultivar *'Benico'* was in the range of 0.86-23.52 mg GAE/g DW and 0.59-205.21 mg TE/g DW of starting material prior SCE-CO_2 and decreased in the following manner: PLE-EtOH > SCE-CO_2 > PLE-Acetone > EAE-Viscozyme. The combined SCE-CO_2, PLE and EAE reduced antioxidant capacity of plant material prior SCE-CO_2 89-99%, showing that suggested biorefining protocol is efficient to remove the major portion of non-polar and polar antioxidatively active constituents. Preliminary phytochemical analysis additionally confirmed the efficiency of SCE-CO_2 to extract cannabinoids from *C. sativa* cultivar *'Benico'.*
5. Both tested CBD (100%) and CBDA (27%) preparations were found non-toxic in CaCo-2 cell model and additionally were shown (mainly, CBDA) partially to correct oxidative stress-induced damages in cells. However, additional studies are required to clarify whether this effect might have a biological relevance in humans.

V. LIST OF LITERATURE REFERENCES

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