Industrial Crops & Products 162 (2021) 113247

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Selective recovery of terpenes, polyphenols and cannabinoids from *Cannabis sativa* L. inflorescences under microwaves

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ARTICLE INFO

Keywords:
Cannabis inflorescences
Terpenes
Cannabinoids
Polyphenols
Microwave-assisted hydrodistillation
Kilo-scale sequential extraction

ABSTRACT

In recent years, hemps health and nutritional properties recognition has led to an impressive growth of *Cannabis* research, industrial processing, and the related market. Moreover, the demand for natural *Cannabis*-derived compounds (i.e. terpenes, polyphenols, and cannabinoids) is constantly growing. In spite of the strict regulation of some countries, the global market needs suitable technologies for the smart recovery of bioactive *Cannabis* metabolites. Conventional extraction procedures can show drawbacks, in terms of environmental impact and their high energy consumption. Microwaves (MW), a mature technique for extraction-process intensification, is attracting great amounts of attention in academic-research and industrial-application fields for its technological advantages. This work aims to design a fast and cost-efficient MW-assisted cascade protocol for bioactive *Cannabis* compounds recovery in a pilot-scale reactor. Microwave-assisted hydrodistillation (MAHD) can provide a volatile hydrodistillate that is rich in monoterpenes, sesquiterpenes, and a small amount of phytocannabinoids. Using non-canonical protocol of hydrodistillation, the definition of "volatile fraction" is generally considered more appropriate than "essential oil".

The health-promoting activity of this combination has been proposed in literature, and can constitute matter of further investigations. The optimized MAHD procedure yielded 0.35 ± 0.02 % w/w of hydrodistillate, while conventional hydrodistillation gave only 0.12 ± 0.01 %, w/w (in relation to dry inflorescence mass). The water resulting in the vessel after MAHD showed a high total polyphenolic content (5.35 ± 0.23 %, w/w). Two flavones known for their beneficial effects to health, namely luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside, were detected and quantified. An attempt to recover phytocannabinoid using the MW-assisted hydrodiffusion and gravity method (MAHG) was also carried out. Cannabinoids (CBD and THC) content was determined in fresh *Cannabis* and in production streams. During MAHD, phytocannabinoid decarboxylation inside the residual matrix was around 70 % (69.01 ± 0.98 % and 74.32 ± 1.02 % for THC and CBD respectively). Furthermore, the overall content of these metabolites was not affected by the hydrodistillation, preserving the processed plant material for subsequent ethanolic extraction.

1. Introduction

Cannabis sativa L. (Cannabaceae family), known as hemp, is a widespread plant species cultivated for a wide range of industrial products (Fathordoobady et al., 2019; Yang et al., 2017; Fiorini et al., 2019). These products are fibres, seed oils, and biomasses that are used in various fields, including in the pharmaceutical, cosmetic, paper, textile, and construction industries, as food and animal-feed additives, phytoremediation agents, biofuel, varnishes, and inks (Fiorini et al., 2019). Hemp has a highly complex chemical composition that includes carbohydrates, terpenoids, alkaloids, stilbenoids, quinones, flavonoids, fatty acids, phenols, and cannabinoids (Brighenti et al., 2017;

Abbreviations: CAR, Cannabimimetic activity receptor; CB, Cotton bag; CBD, Cannabidiol; CBDA, Cannabidiolic acid; CHD, Conventional hydrodistillation; GAE, Gallic acid equivalents; MAE, Microwave-assisted extraction; MAHD, Microwave-assisted hydrodistillation; MAHG, Microwave-assisted hydrodiffusion and gravity method; MW, Microwaves; PEEK, Polyether ether ketone; PTFE, Polytetrafluoroethylene; RT, room temperature; $scCO_2$, Supercritical CO_2; SPE, Solid-phase extraction; THC, Δ^9-tetrahydrocannabinol; THCA, Δ^9-tetrahydrocannabinolic acid; TPC, Total phenolic content; US, Ultrasound.

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https://doi.org/10.1016/j.indcrop.2021.113247
Received 16 August 2020; Received in revised form 6 January 2021; Accepted 6 January 2021
Available online 4 February 2021

Brenneisen, 2007; Drinić et al., 2020). The latter are particular *Cannabis* plant metabolites (Brighenti et al., 2017; Lewis-Bakker et al., 2019). The term phytocannabinoids was proposed for specific *Cannabis* plant products due to the occurrence of synthetic cannabinoids and endocannabinoids (Brenneisen, 2007). One of the most interesting phytocannabinoids in hemp is the non-psychoactive cannabidiol (CBD) (De Vita et al., 2019) whose global market increased to a value of USD 1.90 billion in 2018, and it is estimated that it will grow by a further 49 % by 2024 (BDS Analytics, 2019). Besides CBD, other notable phytocannabinoids that possess no or low psychotropic activity are cannabigerol, cannabichromene, cannabinol, cannabicyclol, cannabinodiol, and there is the psychoactive Δ^9-tetrahydrocannabinol (THC) (Fathordoobady et al., 2019; McAllister et al., 2015).

In recent years, the popularity of medical *Cannabis* extracts has grown rapidly due to extensive reviews of the pharmacological activity of this plant material (Lewis-Bakker et al., 2019), which is mainly attributed to the presence of phytocannabinoids. They act as antiepileptic, anticonvulsive, anti-neurodegenerative, antiemetic, and analgesic agents, and possess antibacterial and anti-inflammatory properties as well (Fathordoobady et al., 2019). Most of these metabolites are present in fresh hemp and carry a carboxylic acid moiety (De Vita et al., 2019; Lewis-Bakker et al., 2019). Acid cannabinoids show low potency for cannabimimetic activity receptor (CAR) binding. However, their decarboxylated homologues forms, usually called neutral cannabinoids, display high affinities for CAR and psychological activities. The decarboxylation step is therefore crucial for the strengthening of *Cannabis* pharmacological activity (Lewis-Bakker et al., 2019), and easily occurs when the acid metabolites are exposed to heat and light, due to their instability (Brighenti et al., 2017; Wang et al., 2016).

The characteristic *Cannabis* fragrance is attributed to approximately 140 different terpenoids (Brenneisen, 2007). In particular, the volatile and semi-volatile fractions in hemp are composed of monoterpenes and sequiterpenes, and some heavier waxes and resins. Additionally, oxygenated terpenoids can also be found (Leghissa et al., 2018). In forthcoming years, terpenoids have received great attention because of their sensorial properties, with peculiar chemical fingerprinting for various *Cannabis* cultivars, and investigations concerning their synergism with phytocannabinoids (Giese et al., 2015). Many studies have proposed the application of extracts, so-called phytocomplexes, containing a mixture of phytocannabinoids and terpenoids, rather than pure synthetic molecules, thus suggesting the existence of complementary or synergistic interactions, often called entourage effects (De Vita et al., 2019; Elzinga et al., 2015). Relative evidences are still to be clarified. In addition, particular terpenoids' pharmacological and medical properties as such have been reported (Fiorini et al., 2019; Leghissa et al., 2018).

The recovery of biologically active compounds, such as phytocannabinoids and terpenes, from hemp is a crucial step for their further applications in the pharmaceutical and food industries (Fathordoobady et al., 2019), and it is typically performed under conventional solid-liquid extractions, such as maceration and percolation. Soxhlet and hydro/steam distillation, entail high energy consumption, long extraction times and can only provide the partial recovery of the desired compounds (Chemat et al., 2012). Over the last decade, attention has shifted to the development of innovative enabling extraction techniques, such as microwave-assisted (MAE), ultrasound (US), pressurized-liquid, supercritical-fluid extraction and instant controlled pressure-drop, with the aim of overcoming these shortcomings (Fathordoobady et al., 2019; Chemat et al., 2019). The use of microwave (MW) technology in bioactive-compound extraction offers a number of advantages: rapid heating, shorter process time, reduction in solvent usage, higher reproducibility, higher extraction rates, and increases in yield (Fathordoobady et al., 2019; Lewis-Bakker et al., 2019; Veggi et al., 2013). Extraction rates and yields, in particular, can be increased by the enhancement of heat and mass-transfer phenomena, working in synergy (Veggi et al., 2013).

Terpenoid yields usually vary from 0.01 to 1.5 % of the inflorescence dry weight (Giese et al., 2015). The hemp volatile fraction, as mentioned, consists of monoterpenes, such as α-pinene, myrcene and terpinolene, and bitter-tasting sesquiterpenes, such as E-caryophyllene, α-humulene, and caryophyllene oxide (Fiorini et al., 2019). These compounds can be recovered via hydro- or steam distillation using Clevenger apparatus, which is the conventional extraction technique, or by means of supercritical CO_2 ($scCO_2$) (Brenneisen, 2007; Markle, 2019). As abovementioned, steam and hydro-distillation have numerous drawbacks and, moreover, their harsh conditions can affect essential-oils quality (Markle, 2019; Lucchesi et al., 2004; Iriti et al., 2006; Ferhat et al., 2007). Supercritical CO_2 extraction protocols are well suited for phytocannibinoids recovery, and improved selectivity can be achieved by varying working pressure by co-solvent additions (Marzorati et al., 2020; Moreno et al. (2020). Nevertheless, the main disadvantage of $scCO_2$ extraction is the fact that processing fresh plant materials is impossible due to the formation of carbonic acid from CO_2 and water (Markle, 2019). The required desiccation of the matrix dramatically affects the whole volatile-composition fingerprint (Fiorini et al., 2019).

Microwave-assisted hydrodistillation (MAHD), can be an efficient alternative for *Cannabis* terpenes recovery. This process is much more efficient than traditional hydro- and steam distillation as the irradiation heats the plant material evenly (Markle, 2019; Ciriminna et al., 2017). Many recently published studies have indicated that MW can even enhance oil extraction, by reducing process time and boosting productivity, when compared with conventional extraction methods (Rezvankhah et al., 2019). Abovementioned advantages of this technique opened the way to its application in the extraction of phytocannabinoids, to date comprehensively reviewed (Brighenti et al., 2017; Lewis-Bakker et al., 2019; Drinić et al., 2020). Microwave-assisted extraction enables phytocannabinoids decarboxylation unlike several other extraction methodologies, where the occurrence of this phenomenon is quite negligible (Brighenti et al., 2017; Lewis-Bakker et al., 2019). This feature is of great importance as it leads to high quality products with measurable pharmacological activity in patients (Lewis-Bakker et al., 2019). In addition to cannabinoids and terpenoids applications, several publications have described hemp polyphenols MAE (Drinić et al., 2020; Matešić et al., 2020; Teh et al., 2014).

2. Material and methods

2.1. Materials

Ethanol (ACS grade, ≥99 %), used for cannabinoid extraction, and methanol (ACS grade, ≥99 %), used for polyphenol enrichment and HPLC analysis, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q H_2O was obtained in the laboratory using a Milli-Q Reference A + System (Merck Millipore, Darmstadt, DE, USA). The standards (Cannabis Terpenes Mix A, Cannabis Terpenes Mix B, cannabidiol, Δ^9-tetrahydrocannabinol, gallic acid, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, catechin, epicatecthin, chlorogenic acid, caffeic acid, quercetin-3-*O*-glucoside), the *Folin–Ciocalteau* reagent and sodium carbonate, for total phenolic assays, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant material and its inflorescence content

The plant material studied was *Cannabis sativa* L. cv. Monoica, and was kindly provided by the company Egeria s.r.l. (Milano, Italy). The matrix was collected in the middle of September 2019 at the fields of *Azienda Agricola Prina Pietro* (Pavia, Italy, N 45°13′10.3″, E 9°11′22.1″, 2.7 ha) and was in a 8.7 phenological growth stage (60 % ripe fruit). After collection, the fresh plant material was vacuum packed and stored at −18 °C. In all experiments, the plant material was used without defrosting.

The collected *Cannabis* contained inflorescences, leaves, and stalks.

One kg of *Cannabis* was thoroughly selected and weighed in order to obtain the ratio between the inflorescence and the other components of the matrix.

2.3. Water-content determination in plant material

The water content in frozen *Cannabis* was determined using the gravimetric method. Plant material was sampled in triplicate from 1 kg frozen bag and dried in a furnace muffler (Gelman Instrument Company, USA) at 100 °C for 24 h.

2.4. Volatile extraction

2.4.1. Microwave-assisted hydrodistillation (MAHD)

The terpene fraction from the *Cannabis* was recovered using MAHD. It was performed in an ETHOS X (Milestone s.r.l., Italy), a multimode MW reactor, at a maximum delivered power of 1800 W (Fig. 1A). All extractions were performed in a 12 L vessel The temperature was monitored using an infrared sensor. The MW power during the extractions was set as follows: 500 W for 3 min, 1100 W for 3 min, 1600 W for 14 min, and finally 1500 W for 90 min. The overall time, necessary to complete volatile compound extraction, was then 1 h and 50 min.

Twelve tests were performed under different conditions. 2.5–2.8 kg of matrix were extracted in all tests. The plant material was always placed evenly in the extraction vessel directly from the freezer.

Even though the *Cannabis* was fresh and still hydrated, supplementary water was placed in the extraction vessel together with the material prior to extraction. The extractions were performed with matrix-to-liquid ratios of 1/0.5–1/1.5 (kg/L). Moreover, the use of tap water, deionized water, and a NaCl solution (20 %) was tested.

Once the vessel was filled with plant material and water, it was placed into the MW cavity of the reactor. The distillation head was assembled with a florentine vase and the extraction process was started. As the terpenes are distilled together with a large amount of liquid, the water was able to recirculate from the florentine vase back into the vessel.

Table 1 reports the mass of extracted *Cannabis*, the plant-material-to-water-ratio and water feed used in every test, as well as the equipment and method alterations made to the processes. As reported in Table 1, additional alterations were made for some tests. In Tests 7, 8, and 9, the plant material was placed in a cotton bag (CB) during extraction. A polyether ether ketone (PEEK) net was placed above the matrix in Test 6. Both the CB and net were used to homogenize the re-circulated water distribution and to enhance overall wetness during extraction, thus helping to prevent the browning effect and potential degradation. In Test 4, the plant material was moved every 30 min, temporary removing the vessel from the chamber. Hot water (50 °C) was added at the beginning of Test 3 to fasten the onset of terpene distillation. In Test 12, a fractionating Vigreux column (20 cm length) was assembled to connect the MW cavity and the distillation head, instead of the regular straight column, in order to investigate the variation in the volatiles fingerprint. Finally, in Test 13, the sampling of the recovered terpenes was performed every 15 min to follow changes in terpene profile with extraction time.

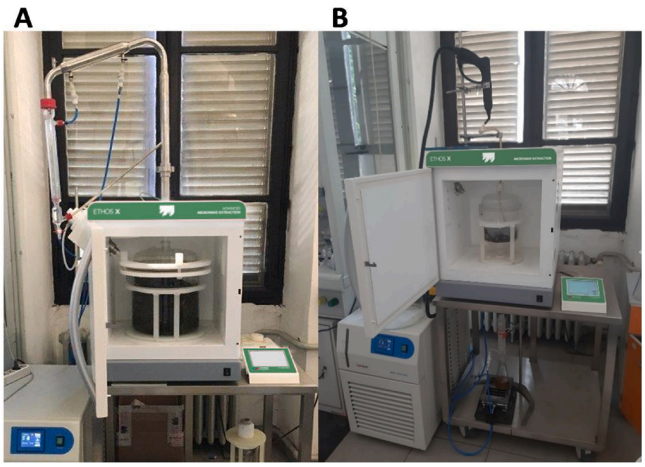

Fig. 1. *Cannabis sativa* L. storage under vacuum.

Once the run was completed, the terpenes fraction was recovered from the florentine vase of the MW system. The extracted terpenes are not miscible with water and hence can be found as the lighter oily phase above the water column. Every run was performed in triplicate and the mass of the obtained volatile fraction was noted for every extraction and expressed as average ± S.D. Yield of the volatile fraction was calculated both in relation to dry matrix and on dry inflorescences. The volatile fraction was then analyzed using GC-MS. The CBD and THC quantitative analysis of the extract that was obtained in the optimal MAHD test was performed using UPLC-MS/MS.

2.4.2. Conventional hydrodistillation (CHD)

Conventional hydrodistillation was performed in order to compare the efficiency of MAHD terpene extraction to a conventionally applied procedure. It was carried out according to the essential oils extraction methods described in European Pharmacopoeia 8th Edition (2013) with few modifications due to the equipment limitations. The *Cannabis* was placed in a 2 L round bottom flask and deionized water was added at a solid/liquid ratio of 1–5. The round bottom flask was placed inside a heating mantle, whilst a Clevenger-type apparatus and a refrigerant were assembled. The extraction time was 4 h. The yield of the recovered hydrodistillate was expressed on dry matrix and only dry inflorescence mass. The hydrodistillate yield and composition was compared with the ones obtained in MAHD tests. The extraction was performed in triplicate, expressing the results as average ± S.D.

2.5. Hydrodistillate analysis

The GC-MS qualitative analyses of the volatile fractions obtained in MAHD and CHD were performed in an Agilent Technologies 6850 Network GC System fitted with a 5973 Network Mass Selective Detector, 7683B Automatic Sampler, and a capillary column Mega 5MS (length 30 m; i.d. 0.25 mm; film thickness 0.25 µm, Mega S.r.l., Italy) according to the method reported by Gunjević et al. (2020). The identification of the individual compounds was performed with two approaches: 1) by comparing the retention time and mass spectrum with standard compounds, 2) by using GC-MS Wiley275 and NIST05 GC libraries from the acquired chromatograms, considering only matching level over 95 %. The summed areas of the relevant peaks were normalized to 100 %. Relative peak areas, calculated as percentages, were used to evaluate extract composition.

2.6. Water after MAHD

Due to the addition of an abundant amount of water to the plant material before MAHD, there is a significant volume of liquid remaining in the extraction vessel after the process. The aqueous fraction was filtered, freeze dried (LyoQuest – 85 lyophilizer, Azbil Telstar Technologies, Spain), and analysed in terms of dry extract yield and polyphenols.

2.6.1. Total phenolic content (TPC) determination

TPC in the water fraction after MAHD was determined according to the method described in Hillis and Swain (1959). 250 µL of the extract solution (1 mg/mL in 50 % EtOH) was placed into the test tube and diluted with 4 mL of deionized water. A sodium carbonate solution (10 %, w/v) and the *Folin–Ciocalteu* reagent (diluted 1:1 with deionized water) were added sequentially. The resulting solution was mixed

Table 1
Cannabis microwave-assisted hydrodistillation tests: screening of parameters, recovered volatile fraction mass and yield, calculated in relation to the complete matrix and based on the only inflorescence, distillation onset time, and cannabidiol (CBD) in the hydrodistillate expressed as percent area in GC-MS chromatogram. Every experiment was performed three times. Results are expressed as average values ± S.D.

Test	Plant material [kg]	Water feed	Plant material to water ratio [kg/L]	Additional process alterations	Hydrodistillate [g]	Yield over complete matrix [a] [%, w/w]	Yield over only inflorescence [a] [%, w/w]	Distillation onset [min]	CBD in hydrodistillate [b] [Area %]
1	2.60	Deionized	1/1	–	1.84 ± 0.10	0.24 ± 0.02	0.33 ± 0.02	16	2.49
2	2.60	Tap water	1/1	–	1.91 ± 0.09	0.24 ± 0.02	0.35 ± 0.02	16	1.75
3	2.64	Deionized	1/1	Hot water added	1.74 ± 0.12	0.22 ± 0.02	0.31 ± 0.02	12	2.40
4	2.70	Deionized	1/1	Matrix moved during the extraction	1.28 ± 0.12	0.16 ± 0.01	0.22 ± 0.02	16	2.34
5	2.72	20 % NaCl	1/1	–	0.66 ± 0.09	0.08 ± 0.01	0.11 ± 0.02	14	10.51
6	2.73	Deionized	1/1	PEEK [c] net above the matrix	1.46 ± 0.08	0.18 ± 0.01	0.25 ± 0.01	16	3.20
7	2.84	Deionized	1/1	Matrix in a cotton bag	1.36 ± 0.11	0.16 ± 0.01	0.22 ± 0.02	19	0.30
8	2.63	Deionized	1/1	Matrix in a cotton bag, hot water	1.26 ± 0.09	0.16 ± 0.01	0.22 ± 0.02	14	0.55
9	2.80	Deionized	1/1.5	Matrix in a cotton bag	1.49 ± 0.13	0.18 ± 0.01	0.25 ± 0.02	19	0.62
10	2.50	Deionized	1/1.5	–	1.37 ± 0.12	0.18 ± 0.01	0.26 ± 0.02	16	4.10
11	2.61	Deionized	1/0.5	–	1.54 ± 0.09	0.20 ± 0.02	0.28 ± 0.02	16	2.77
12	2.74	Deionized	1/1	Rectification with Vigreux column	1.58 ± 0.09	0.19 ± 0.01	0.27 ± 0.02	15	2.21
13[d]	2.69	Deionized	1/1	–	–	–	–	–	–

[a] yields expressed on dry matter.
[b] GC-MS relative area.
[c] PEEK - Polyether ether ketone.
[d] no results reported since this extraction was performed to evaluate the composition of the volatile fraction during the extraction by periodical sampling.

thoroughly. After 25 min, the absorption of the blue complex was measured at 725 nm, in a 1 cm quartz cuvette, using a Cary 60 UV–vis spectrophotometer (Agilent Technologies, USA), against a blank. Gallic acid was used as the standard. TPC was expressed as gallic acid equivalents (GAE, mg/g) over the dried extract and gallic acid equivalents (GAE, mg/g) over the dried matrix. All analyses were performed in triplicate.

2.6.2. Polyphenol enrichment

Polyphenols from the water fraction after the optimal MAHD protocol were enriched using solid phase extraction (SPE) on a C18 Sep-Pak cartridge (Waters, USA) for analytical purposes, following the procedure described by Gunjević et al. (2020). Purified polyphenolic rich fraction was analysed using HPLC-DAD.

2.6.3. Polyphenol analysis

Identification and quantification of polyphenols present in the above described fraction (paragraph 2.6.2.) were performed using a HPLC system (Waters Corp., USA) coupled with a diode array detector (UV/DAD, Waters Corp., USA) and an automatic sampler (Waters Corp., USA). In particular, luetolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, catechin, epicatecthin, chlorogenic acid, caffeic acid, and quercetin-3-*O*-glucoside separation was achieved on a Synergi Hydro RP C18 column (250 mm, 4.6 mm, 5 µm; Phenomenex, USA) by gradient elution and UV-DAD acquisitions as described by Gunjević et al. (2020). Chromatograms were acquired at 340 nm, performing three injections for each sample.

2.7. Phytocannabinoid extraction

Phytocannabinoids were extracted both from the fresh matrix and from the depleted biomass in the optimal MAHD process. Cannabinoid extraction from fresh plant material was performed for two purposes. The first objective was the fresh plant determination of CBD and THC content. The second target was to provide a control parameter for cannabinoid decarboxylation after MAHD. Together with the conventional benchmark, a MW-assisted protocol was tested on the fresh plant to investigate the technique's phytocannabinoid-recovery efficiency. The *Cannabis* inflorescence was separated from the rest of the plant in every extraction.

2.7.1. Conventional extraction under reflux

For analytical purposes, conventional reflux cannabinoid extractions were performed using ethanol (99 %) as the solvent. The extraction time was 2.5 h and the solid-to-liquid ratio was 1–10. The obtained extract was filtered and the ethanol was evaporated. Moreover, two extractions were performed for every sample to evaluate the decarboxylation efficiency of the MAHD extraction method. In one of these extractions, the *Cannabis* inflorescence was placed in a furnace muffler for 30 min at 120 °C before extraction to promote cannabinoid decarboxylation, while this step was skipped in the other extraction. For every obtained extract, the yield was noted and the CBD, CBDA, THC, and THCA contents were evaluated. Every set of extractions was performed in triplicate and the contents of CBD, THC and their acid analogues were expressed as average ± S.D.

2.7.2. Microwave-assisted hydrodiffusion and gravity (MAHG)

Microwave-assisted hydrodiffusion and gravity was also performed in the ETHOS X MW reactor, but using a different system configuration in which the extract was recovered in the flask placed under the reactor (see Fig. 1B). The frozen Cannabis (200 g) was placed evenly in the 5 L extraction vessel, which was housed in the MW cavity of the reactor. The condensation system and the collection flask were assembled from the bottom of the device. Thanks to the opening on the top of the MW cavity, steam was introduced into the system. The extraction method provided a continuous irradiation of 200 W for 60 min. Steam was fed into the vessel for 30 s every 5 min. The temperature was monitored with an infrared sensor and never exceeded 100 °C.

During the extraction, the extract was continuously collected in the receiving flask. Once the process was completed, the collected extract was freeze-dried and analyzed for its extraction yield, and CBD and THC

content. This extraction was performed 3 times, and the extraction yield, CBD and THC contents were expressed as average ± S.D.

2.8. Phytocannabinoid analysis

Quantitative determination of phytocannabinoids CBD and THC was carried out on a Waters Acquity TQD UPLC-MS/MS system, Using a Waters BEH C18 (2.1 × 50, 1.7μ) column. Adopted method and relative calibrations are reported by Gunjević et al. (2020). Each sample was divided in two specimens: the first was directly analysed whilst the second was firstly decarboxylated in oven. THCA and CBDA were quantified as difference between cannabinoids detected in the two specimens.

2.9. Statistical analysis

Statistical data analysis was performed using software Statistica (Statsoft Inc., Tulsa, OK, USA), v.10. The measurements were processed using Tukey's HSD test and statistical differences (p-value < 0.05) were indicated by lower-case letters on the Figures.

3. Results and discussion

3.1. Inflorescence and water content in plant material

The collected *Cannabis sativa* L. cv. Monoica consisted of 73.70 % ± 3.22 % w/w of inflorescence and 36.30 % ± 2.98 % w/w of stalks and leaves.

The average water content in *Cannabis*, determined by thermogravimetric analysis, amounted in 69.97 ± 2.63 %, w/w. In particular, 71.15 ± 0.98 % was in the inflorescences, while 59.72 ± 0.89 %, w/w in separated stalks and leaves.

3.2. Volatiles extraction

3.2.1. Microwave-assisted hydrodistillation (MAHD)

By considering the growing demand for *Cannabis*-derived terpenes from today's hemp market, the aim of this work is to present a novel pilot-scale extraction procedure for their recovery. Extractions were performed in a multimodal MW reactor and several tests with different extraction conditions were investigated (see Table 1).

The terpene-fraction mass was monitored for each test (GC-MS percentage peak area). Moreover, CBD relative area % was registered, as a control parameter to describe pyhtocannabinoids extraction behaviour. CBD was conveniently chosen being the most abundant in the matrix. The hydrodistillate mass and yield, the time of distillation onset, and CBD are reported for every MAHD test in Table 1.

First, the quantity of water added to the system to enhance the stripping power of steam was screened, and its influence on the process was determined. Water addition can increase terpene yield but, more importantly, it prevents partial material burning (consequently, metabolites degradation), thus preserving quality and use of the matrix after MAHD, such as selective phytocannabinoids recovery (Markle, 2019). Moreover, material combustion during distillation can lead to the release of undesired compounds into the volatile fraction. All sources of water (added and contained in the plant) were heated during the extraction, generating steam that allows the release of terpenes from *Cannabis* inflorescence and carries them to the distillation head. As reported in Fig. 2A, the intermediate plant/liquid ratio of 1:1 proved to be the most efficient, as it kept the matrix wet until the end of the extraction and led to the highest yield. For this reason, the remaining MAHD-screening tests were carried out using this water amount. Fiorini

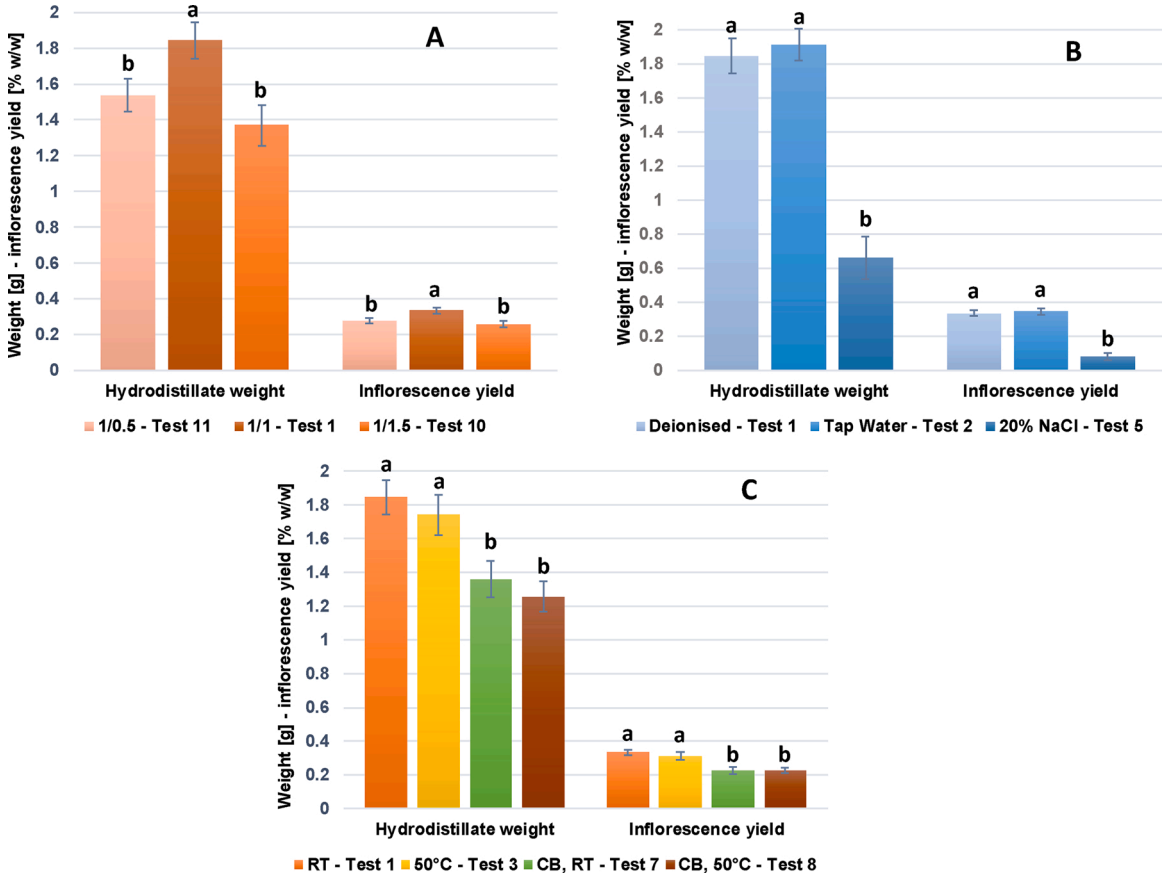

Fig. 2. ETHOS X, MAHD set-up.

et al. (2020) performed MAHD in a similar reactor set-up, and likewise studied the water addition effect. These authors reported the highest volatiles yield when 30 % of water was added. Further addition of water decreased the yield. This consideration differ much from results reported in Fig. 2A, according to which, when expressing the water addition in percentage, the highest volatile fraction's yield was achieved when water content was 50 %, while yields decrease was noted for both 25 % and 75 % water contents.

Moreover, the effect of having a deionized water (Test 1) or feed with different quantities of solutes, namely tap water and a 20 % $NaCl_{aq}$ solution (Test 2 and Test 5), was studied. The greatest yield was observed in Test 2, followed by Test 1 and Test 5, as shown by comparison reported in Fig. 2B. Yields from Test 1 and Test 2 were not statistically different. However, since tap water does not require additional treatments as the deionization, it was selected. A high amount of salts is usually exploited to enhance MW absorption, hence leading to higher temperatures and a faster heating ramp. In fact, the onset of distillation was reduced by 2 min for Test 5. However, the rapid temperature increase led to the lowest extraction yield observed, instead of increasing hydrodistillate recovery. Compound degradation, likely due to the increased boiling point of the system and difficult temperature control, is assumed to be the reason (Mcgraw et al., 1999; Namdar et al., 2018). Nevertheless, this peculiar episode requires further study.

The use of hot water (50 °C) as the liquid feed was considered as a mean to accelerate the distillation onset, while investigating how this approach could affect the extraction of volatiles. This approach could allow to speed up the distillation onset, reducing the MW irradiation time on the plant material. Thus, the matrix can be preserved from degradation phenomena. As expected, MAHD onset was accelerated from 16 to 12 min, saving a quarter of the total heating step (see Table 1, Test 2 *vs.* Test 3). As depicted in Fig. 2C, both the volatile fraction and inflorescence yield were slightly affected by the hot-water protocol, however not statistically significant. It can be assumed that the products leaked during addition and plant preparation because of the high volatility of the terpenic compounds. Since no statistical difference was noted, remaining tests were performed with room- temperature (RT) water addition.

To prevent any loss during matrix moisturizing and positioning, the same screening was studied using a CB, and both RT and a 50 °C water feed were evaluated. At the same time, the use of a CB had the role of protecting the hemp from overheating, maintaining high wetness, and avoiding burning phenomena. Generally, as reported in Fig. 2C, the use of a CB significantly reduced the average yield of the process showing that the cotton fibres had a quenching effect. Furthermore, the onset of MAHD was significantly delayed, from 16 to 19 min. A similar approach was tested with a PEEK net (Test 6), aiming to evenly distribute the recycled water on the matrix, during the distillation process. Though, also this system led to a decrease in the volatile fraction and inflorescence yield. Test 4 was performed with the matrix being moved every 30 min during extraction. The initial hypothesis was that this should increase the volatile fraction yield, compensating eventual temperature inhomogeneity, hence releasing terpenes contained in every spot of the matrix. On the other hand, the extraction yield was much lower. The explanation of this obtained result can be related to the necessary equipment extraction and dissembling, in order to carry out the manual matrix movement, that lead to a volatile-compound loss.

Close attention was paid on the state of the vegetal matrix after the extraction treatment, to evaluate any biomass overheating or burning effect. This never happened, even when the plant material was placed in a CB for MAHD. In this case, the matrix appeared to be driest between the screened conditions. Generally, it is possible to state that the hemp that resulted from the MAHD was preserved from combustion and degradation phenomena, thus it may be suitable for additional extraction. For this reason, the phytocannabinoid decarboxylation after MW irradiation was investigated.

Ethanol extraction under reflux is considered to be the benchmark cannabinoid extraction procedure. Hence, every sample was extracted according to this approach in duplicate, either with a prior heating step of the sample at 120 °C, or directly. The heating protocol was applied to promote acidic cannabinoid decarboxylation. Both fresh *Cannabis* inflorescence and the spent matrix after MAHD were used. The benchmark phytocannabinoid extraction of fresh plant material enabled CBD quantification by means of UPLC-MS/MS analyses. Similarly, THC was monitored and quantified on the base of national regulation on psychotropic substances. According to the most recent regulation in the Italian legislation (note published by the Ministry of the Interior 20/07/2018 number of protocol 2018/43586), commercial uses of resins, concentrates and essences (or inflorescences and plants) with THC concentrations >0.5 %, are considered illegal substances. Hence, detention and commercialization are illegal (DPR 309/90). Given the abovementioned regulations, it is mandatory to have a suitable analytical method for THC determination to verify the conformity. UPLC-MS/MS results are summarized in Table 2.

The final analysis of the matrix after MAHD confirmed that MW irradiation gave phytocannabinoid decarboxylation of about 70 % of the total (69.01 ± 0.98 % and 74.32 ± 1.02 % for THC and CBD, respectively). As already mentioned, MW enables extensive phytocannabinoids decarboxylation, providing more active forms of cannabinoids (Lewis-Bakker et al., 2019), hence it can be considered for further investigations.

Percent area of CBD in the volatile fractions was carefully monitored using GC-MS, as a control parameter for phytocannabinoids state in the hydrodistillate, due to their biological activity. Fig. 3 compares the CBD trend to hydrodistillate yields, as calculated on only dry inflorescence. MAHD provides efficient hydrodistillate recovery and good phytocannabinoid decarboxylation before residual matrix extraction with ethanol. Nevertheless, it does not deplete the matrix of phytocannabinoids. Hence, the optimized protocol should maximize terpenoids yield and preserve CBD for the next step.

The screening of different plant/water ratios allowed achieving the lowest CBD relative area at a 1/1 ratio (Test 1), while, unlike what Fiorini et al. (2020) observed, this significantly increased with liquid content increase (Test 10, Fig. 4A). Moreover, the liquid content reduction to 1/0.5 ratio led to a limited but statistically significant increase in CBD area, when compared to the 1/1 ratio.

An even more pronounced increase in this cannabinoid was detected using 20 % $NaCl_{aq}$ MAHD (Test 5), which yielded in the highest CBD percent area, with 10.51 % vs. 2.49 % and 1.75 % using deionized and tap water, respectively (Fig. 4B). This trend can be explained by the increase in the water boiling point, thus permitting the distillation of compounds with lower volatility.

Changing the water-feed temperature did not noticeably alter the CBD area in GC-MS chromatogram of the hydrodistillate, although there was a slight decrease at 50 °C (Test 1 and Test 3, Fig. 4C). On the other hand, the cannabinoid area was dramatically lower, namely 0.3, 0.55,

Table 2
Cannabidiol (CBD), cannabidiolic acid (CBDA), Δ^9-tetrahydrocannabinol (THC) and Δ^9-tetrahydrocannabinolic acid (THCA) UPLC-MS/MS quantification. Raw inflorescence: percentage concentrations for acidic and decarboxylated cannabinoids. Test 2 hydrodistillate and inflorescence depletion: decarboxylated and acid forms reported as total amount; result expressed as ratio between the cannabinoid (both forms) content and the cannabinoid content (both forms) in fresh inflorescence. Every experiment was performed 3 times. Values are expressed as average values ± S.D.

	Inflorescence content [%, w/w]	Hydrodistillate content [%] [a]	Inflorescence Depletion [%] [a]
THC	0.02 ± 0.004	0.04 ± 0.005	0.07 ± 0.006
THCA	0.05 ± 0.005		
CBD	0.34 ± 0.02	0.42 ± 0.03	0.05 ± 0.004
CBDA	0.66 ± 0.04		

[a] Test 2 analysis; expressed as total amount of decarboxylated and acid forms.

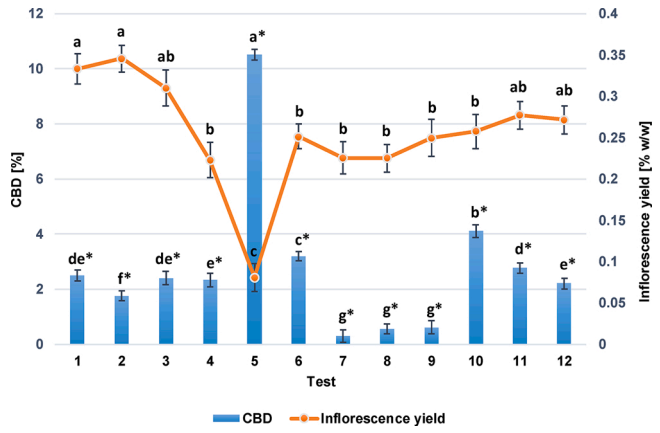

Fig. 3. ETHOS X, MAHG set-up.

and 0.62 % for Tests 7, 8, and 9, when the hemp was placed in a CB. The other physical barrier used, a PEEK net placed above the matrix (Test 6), gave higher CBD area on the hydrodistillate GC-MS chromatogram, more precisely 3.20 %. Plant material movement during the extraction (Test 4) did not affect the CBD percent area (Test 1). In Test 12, a fractionating Vigreux column was assembled to connect the extraction vessel with the distillation head. The Vigreux column permits volatile compounds to be separated by allowing the vapours to cool, condense, and vaporize again. Every condensation-vaporization cycle enriches vapours in a certain component, and the larger surface area of the Vigreux column allows more cycles to be performed (Zuiderweg and Harmens, 1958). Therefore, this set-up has the objective of distilling the low boiling point terpenes and separating them from the high boiling point cannabinoids. However, CBD area in the obtained volatile fraction chromatogram was 2.21 %, which is analogous with the result obtained in Test 1, where a regular straight column was used.

The analytical data indicate that Test 2 gave the best results, allowing to the highest volatiles yield when performed with tap water, which is preferable on pilot and industrial scales. The yield of volatiles fraction expressed on the whole dry matrix was 0.24 ± 0.02 % (w/w), which corresponded to 0.35 ± 0.02 % (w/w) calculated in relation to only dry inflorescence. The effective cannabinoid content of the hydrodistillate, finally, was evaluated by means of UPLC-MS/MS. Results are reported in Table 2, and define a negligible depletion of the plant material from these metabolites, resulting in nearly unaffected inflorescence.

3.2.2. *Conventional hydrodistillation (CHD)*

Conventional hydrodistillation was performed in order to compare the volatile fraction yield, and its terpene profile, with the one derived from a non-conventional extraction procedure, MAHD. The recovered hydrodistillate yield obtained in this process was 0.12 ± 0.01 %, w/w, as calculated in relation to the only dry inflorescence, and 0.08 ± 0.01 %, w/w, when calculated on the whole dry matrix. Production was hence about 3 times lower than the one obtained in the optimized MAHD, by applying an extraction time of 4 h, therefore significantly longer. Moreover, CHD was performed using conventional conductive heating, which is inefficient and has high energy consumption due to thermal dispersion and material calorimetric restrictions. The slow conductive heating means that the onset of terpene extraction was heavily delayed compared with MAHD. These results confirm that process intensification occurred when MW was applied.

As showed by GC-MS analyses, the CBD percent area in the resulting volatile fraction chromatogram was 23.83 %, ergo about 14 times higher than in the volatile fraction derived from the optimal MAHD test. Fiorini et al. (2020) likewise noted higher CBD yield in CHD volatile fraction,

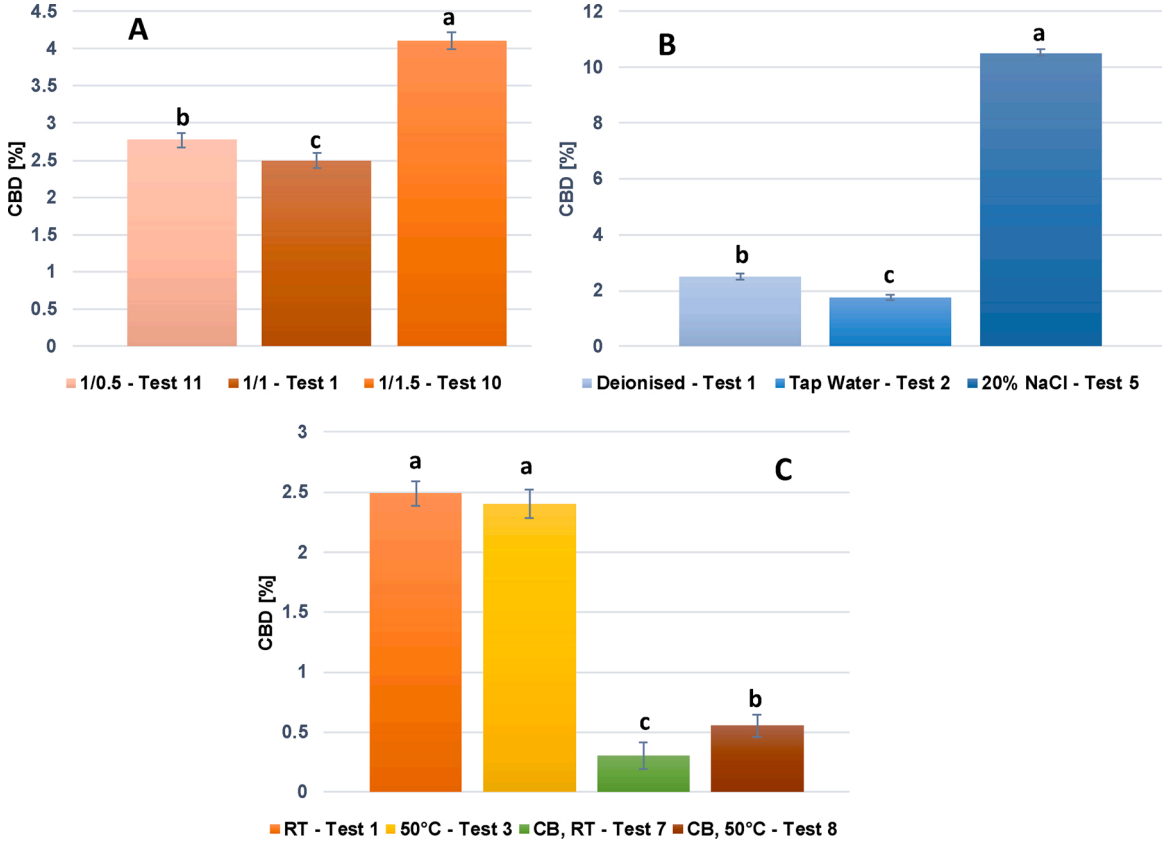

Fig. 4. MAHD trend with different water feeds (plant/liquid ratio w/w:1/0.5, 1/1, 1/1,5). Data on hydrodistillate weight (g) and only inflorescence (DM) yield (%, w/w).

when compared to MAHD. The analysed sample showed traces of THC as well, proving the harshness of the protocol. Considering the very low yield in the desired volatile fraction, the residual water was not tested for polyphenolic content.

3.3. Volatile fraction analysis

3.3.1. Volatiles from MAHD

A qualitative analysis of the terpenes was performed using the GC-MS system, matching 93.6 % of the overall composition by the comparison with standard compounds and mass spectra libraries (quality ≥95 %). The non-assigned compounds show very low area percentages and poor libraries quality matching (<<95 %). Hence, they were assumed to be barely significant. The compounds contained in the sample that was obtained from the optimized MAHD Test 2 are listed in Table 3, and are expressed as relative peak areas on the GC-MS chromatogram. A detailed report of retention times and mass fragmentations for every detected compound is reported by Gunjević et al. (2020). Whereas the relative percent area of CBD has already been reported in the paragraph 3.3.1., it was not shown in Table 3.

As can be seen from Table 3, the prevailing terpenoids with highest peak areas are as follows: monoterpenes: α-pinene, β-myrcene, β-ocimene; and sesquiterpenes: E-caryophyllene, α-humulene, caryophyllene oxide, and β-selinene. These are the compounds typically present in the volatile hydrodistillate of European *Cannabis sativa* L. (Brenneisen, 2007). α-Pinene has a characteristic pine fragrance and exhibits antiseptic properties. β-Myrcene is characterized by a musky fragrance as well as antioxidant and chemo-protective effects. Caryophyllene has a peppery fragrance, and gastro-protective and anti-inflammatory biological activity (Leghissa et al., 2018). Moreover, it is a Food and Drug Administration (FDA) approved food additive. Caryophyllene oxide is used as the marker compound for marijuana detection by trained dogs

Table 3
Terpene fraction profile obtained in microwave-assisted hydrodisillation Test 2. Values expressed as normalized percent peak area composition obtained from GC-MS analysis.

Volatile fraction profile			
Compound	Area %	Compound	Area %
α-thujene[b]	0.32	α-Copaene[b]	0.19
α-pinene[a]	10.78	*Z*-caryophyllene[b]	0.66
Camphene[a]	1.65	α-*trans*-bergamotene[b]	0.40
Sabinene[b]	0.12	*E*-caryophyllene[a]	8.91
β-pinene[a]	4.09	β-farnesene[b]	1.82
β-myrcene[b]	6.74	α-humulene[a]	4.32
δ-3-carene[a]	3.55	β-patchoulene[b]	0.95
α-terpinene[a]	0.19	β-selinene[b]	4.22
o-cymene[b]	0.08	α-selinene[b]	2.88
Limonene[a]	1.82	δ-cadinene[b]	1.78
1,8-cineole[b]	1.16	α-gurjunene[b]	2.46
β-ocimene[b]	7.02	Aromadendrene[b]	2.65
γ-terpinene[a]	0.28	Selina-3,7(11)-diene[b]	3.32
trans-sabinene hydrate[b]	0.14	Nerolidol[a]	1.95
α-terpinolene[a]	2.55	Germacrene B[b]	2.69
p-cymene[a]	0.04	Caryophyllene oxide[b]	4.93
Dehydro-linalool[a]	0.13	Allo-aromadendrene[b]	0.54
cis-sabinene hydrate[b]	0.07	7-epi-α-selinene[b]	1.41
Fenchol[a]	0.08	caryophylla-4(12),8(13)-diene-5-β-ol[b]	1.84
Pinocarvone[b]	0.04	α-bisabolol[a]	0.94
Borneol[a]	0.05	Eudesm-7(11)-en-4-ol	0.29
Terpinen-4-ol[b]	0.15	Hexahydrofarnesyl acetone[b]	0.11
α-terpineol[a]	0.11	Heptacosane[b]	0.02
n-Tridecane[b]	0.06	Nonacosane[b]	0.07
α-ylangene [b]	0.14		

[a] Identified according to standard compound.
[b] Assessed according to Wiley275 and NIST05 GC libraries (matching quality ≥ 95 %).

(Fiorini et al., 2019).

Fiorini et al. (2020) performed MAHD of *Cannabis* volatiles, obtaining a CBD enriched volatile fraction. The main components present in this extract were caryophyllene, CBD, α-humulene, α-pinene, caryophyllene oxide and myrcene. Therefore, the recovered terpenes composition is similar to Test 2 extract, even if composition deviations are observed.

As already mentioned, the extraction that gave the highest hydrodistillate yield (Test 2) was repeated in order to observe how the composition profile evolves during extraction (Test 13). The terpenic fraction was sampled six times after the onset of distillation. After sampling, the florentine vase was thoroughly washed with acetone and water to avoid the remaining compounds interfering with the following sample. No significant changes in general terpene trend in relation to extraction time were noted. However, the trend of the percent areas of the main monoterpenes (α-pinene, β-myrcene, β-ocimene) and sesquiterpenes (E-caryophyllene, α-humulene, and caryophyllene oxide) was investigated (see Table 4 and Fig. 5) at each sampling time. For the sake of comparison, percentage areas were normalized exclusively in relation to the abovementioned compounds.

On Fig. 5 it can be seen that on average, the monoterpenes relative area constantly increased according to extraction time, while the E-caryophyllene, α-humulene, and caryophyllene oxide area decreased; during extraction, monoterpene area overtakes the decreasing sesquiterpene percent area. Nevertheless, lighter terpenes were the most abundant compounds in all of the analysed samples. Subsequently, all of the fractions were united and analysed by GC-MS to verify the overall composition in respect to the Test 2. The prevalent terpenes percent areas were found to be quite comparable with the terpenes from the volatile fraction that was obtained in the optimal MAHD test.

The decreasing trend of sesquiterpene relative area during extraction may be related to the progressive depletion of the matrix, as the lower quantity of these compounds in hemp inflorescences is well known (Aizpurua-Olaizola et al., 2016; Booth et al., 2017). Monoterpenes, which are usually predominant, are even more pronounced in the extracted matrix, due to the post-harvesting strategies. CBD, whose percent area trend is shown for every sample in Fig. 5, was found to be present across all the sampling times, with correlated percent area changes during extraction. The reported plot shows a gradual increase in CBD area on GC-MS chromatogram over extraction time. When the sampled fractions were combined, the CBD percent area was 2.29 %, which is comparable to the optimal MAHD test.

3.3.2. Volatiles from CHD

The *Cannabis* volatile fraction profile obtained by means of CHD is reported in Table 5. Since the percent area of CBD in CHD extract's chromatogram has already been reported in the paragraph 3.2.2., it was not shown in this Table. Predominant compounds found in the gas-chromatographic profile include: E-caryophyllene, caryophyllene oxide, α-humulene, β-selinene, and α-bisabolol. It is immediately clear that terpenoid fraction is characterized by a reduced variety, if compared with MAHD product. More in detail, a much higher contribution of sesquiterpenes is observed. However, this highlights how for a vegetable matrix like *Cannabis*, which possesses a little essential oil content, better extractive yields in volatile compounds, such as terpenes and sesquiterpenes, can be obtained thanks to the action of unconventional techniques such as MW. Tested MAHD allows process intensification by shortening extraction time, thus avoiding the loss of volatile compounds and secondary metabolite degradation. Therefore, terpene profile does not only depend on *Cannabis sativa* variety, growth stage, and cultivation position, but also on the extraction method.

Gulluni et al. (2018) analysed *Cannabis* essential oil belonging to the same variety studied in this work (*Cannabis sativa* L. cv. Monoica), prepared through CHD. The essential oil's prevalent compounds, in particular myrcene, terpinolene, caryophyllene, β-humulene, β-ocimene, and limonene, indicate a slightly different composition with what

Table 4
Percentage relative area of main monoterpenes and sesquiterpenes in the hydrodistillate sampled at different times during Test 13 extraction. Values expressed as percent peak area composition obtained from GC-MS analysis, normalized on the reported compounds.

Compound	Terpene area [%]						
	30 min	45 min	60 min	75 min	90 min	110 min	Total
Monoterpenes							
α-pinene	21.26	22.80	29.65	33.40	37.86	33.94	29.18
β-myrcene	9.51	9.23	11.25	14.12	15.75	17.93	12.17
β-ocimene	15.28	11.49	13.41	15.95	18.18	20.43	15.03
Sesquiterpenes							
E-caryophyllene	32.91	32.95	25.64	20.92	16.70	16.55	26.93
α-humulene	13.21	13.22	10.32	8.05	6.04	5.94	9.41
caryophyllene oxide	7.82	10.31	9.74	7.56	5.48	5.21	7.27

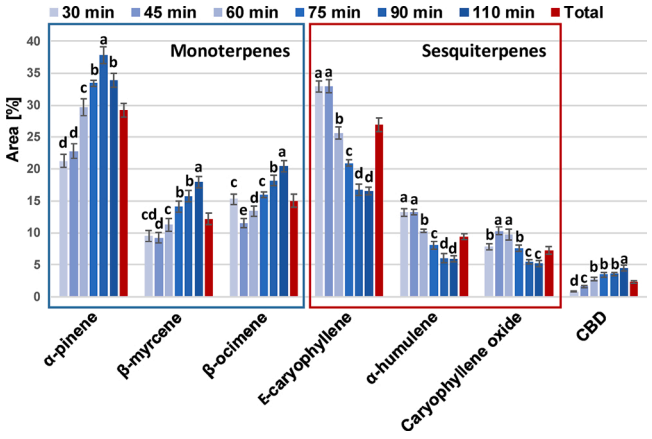

Fig. 5. MAHD trend for different water feeds (deionized, tap water, 20 % NaCl). Data on hydrodistillation weight (g) and only inflorescence (DM) yield (%, w/w).

Table 5
Volatile fraction profile obtained in conventional hydrodistillation. Values expressed as normalized percent peak area composition obtained from GC-MS analysis.

Terpene fraction profile			
Compound	Area %	Compound	Area %
Z-caryophyllene[b]	0.19	α-gurjunene[b]	2.26
α-*trans*-bergamotene[b]	1.49	Selina-3,7(11)-diene[b]	2.09
α-santalene[b]	0.17	Nerolidol[a]	2.53
E-caryophyllene[a]	8.94	Germacrene B[b]	2.82
α-guaiene[b]	0.24	γ-muurolene[b]	0.62
β-farnesene[b]	1.94	Caryophyllene oxide[b]	8.15
Aromadendrene[b]	2.22	Valencene[b]	2.40
α-humulene[a]	4.15	caryophylla-4(12),8(13)-diene-5-β-ol[b]	2.98
β-gurjunene[b]	0.87	α-bisabolol[a]	4.01
γ-selinene[b]	0.89	Eudesm-7(11)-en-4-ol[b]	1.43
β-selinene[b]	6.12	Heptacosane[b]	0.17
α-selinene[b]	3.22	Nonacosane[b]	0.45
β-guaiene[b]	1.82		

[a] Identified according to the standard compound.
[b] Assessed according to Wiley275 and NIST05 GC libraries (matching quality ≥ 95 %).

reported here.

Similarly to MAHD test, the CBD percent peak area (from GC-MS) has been exploited to express the selectivity of volatiles extraction by CHD, surprisingly being 23.83 %. This value indicates a higher amount of phytocannabinoids in the essential oil in respect to the MAHD volatile fraction, thus may limit the applicability of the CHD product.

3.4. Water after MAHD – analysis

The water added to the *Cannabis* plant material before the extraction was, in the most cases, around 2.5 L in quantity. As is already known, MW solid/liquid extraction is widely used in the field of green extraction (Chemat et al., 2019). Polyphenols are some of today's most interesting phytochemicals. They possess several biological effects, including antioxidant, antibacterial, anti-inflammatory, and chemo-preventive power (Cravotto et al., 2018). The MW-assisted extraction of hemp polyphenols has been described in several papers. Teh et al. (2014) have investigated the use of MW as a prior step to US-assisted polyphenol extraction from defatted hemp seed cake. The results have shown that the irradiation of this residue can enhance the metabolites extraction, maximizing the polyphenols yield. Drinić et al. (2020) unified the aforementioned approaches, developing an optimization of hemp MAE for phenols, flavonoids, and phytocannabinoids in ethanol. In this case, MAE was found to be a simple, fast, and efficient extraction method for the cited classes of metabolites, preserving at the same time the high antioxidant activity of the extract.

Therefore, the liquid fraction after the optimized MAHD Test 2 was analysed in order to evaluate its total polyphenols content (TPC). This value, estimated using the *Folin–Ciocalteu* test, was found to be 1.49 ± 0.02 mg polyphenols/g matrix and 53.54 ± 2.35 mg polyphenols/g extract. Hence, the obtained extract contained 5.35 ± 0.23 %, w/w of polyphenols.

3.4.1. Polyphenol enrichment and HPLC analysis

The SPE was used to purify polyphenolic water fraction obtained from optimized MAHD for the sake of analysis. The concentrated polyphenol fraction yield reached 10.31 ± 0.45 %, w/w, calculated in relation to the dry raw extract. The process led to an overall TPC content of 51.71 ± 2.25 %, w/w, calculated in relation to the dry purified sample, achieving a nearly 10-fold metabolite concentration compared with the raw dry extract.

Literature suggests that the main polyphenols in *Cannabis sativa* L. are flavonoids (Koltai and Namdar, 2020; Nagy et al., 2019). Nevertheless, phenolic acids have also been detected in *Cannabis* plant (Izzo et al., 2020). Therefore, HPLC-DAD was used to analyse polyphenols belonging to the aforesaid classes present in the enriched sample. Two main peaks were identified, on the basis of standard compounds, as flavone products, namely luteolin-7-O-glucoside and apigenin-7-O-glucoside, with an amount of 2.84 ± 0.12 %, w/w and 2.58 ± 0.11 %, w/w, respectively, when calculated in relation to the dry purified water fraction. Moreover, the absorption spectrum of each compound detected was thoroughly revised. In particular, six signals, besides luteolin and apigenin glucosides, were detected at 340 nm and featured a spectrum that is characteristic for flavones, as reported in Gunjević et al. (2020). However, the lack of specific standards means that identification and quantification were impossible. In agreement with previous investigations the main polyphenols in low-THC *Cannabis* cultivars were flavones (Brenneisen, 2007).

Thanks to their additive nutritional value, flavones have received

increased attention in recent years. Their main activity is their ability to scavenge oxygen species that contain free radicals that cause oxidative stress (Jiang et al., 2016). Moreover, their beneficial effects on the prevention of cardiovascular, cerebrovascular, and some other chronic diseases, such as asthma, cataracts, diabetes, and rheumatoid arthritis, have been reported (Graf et al., 2005).

The global polyphenol market was valued at USD 1.28 billion in 2018 and is expected to grow by 7.2 % from 2019 to 2025 (GVR, 2019), therefore, this by-product of MAHD can be considered as a valuable and cheap source for the isolation of some of these natural compounds.

3.5. Cannabinoid MAHG extraction

Reflux is the conventional extraction method to achieve cannabinoids recovery from *Cannabis* inflorescence (Baranauskaite et al., 2020; De Vita et al., 2020). It entails a long extraction time and ineffective conductive heating. Moreover, it requires ethanol, a widely used but potentially flammable solvent. A preliminary MAHG test was performed to evaluate the possibility of overcoming the disadvantages of the classical extraction approach. Steam was introduced into the MW cavity to enhance the extraction efficiency by the continuous stripping and to supply water onto plant material. The fresh inflorescence MAHG gave a dry extract of 4.65 ± 0.26 % w/w on dry inflorescence, with CBD and THC content of 0.008 ± 0.001 % and 0.001 ± 0.001 %. These results correspond to a CBD and THC extraction yield of 0.01 ± 0.001 % and 0.04 ± 0.005 %, respectively, when expressed as matrix depletion ratio. Therefore, the matrix depletion of cannabinoids is inefficient when MAHG is applied.

4. Conclusion

More than 2.5 kg per cycle of *Cannabis* plant material was efficiently processed by MAHD in a 12 L Pyrex® vessel. The hydrodistilled oil was extremely rich in the characteristic *Cannabis* terpenes: α-pinene, β-myrcene, β-ocimene, E-caryophyllene, α-humulene, caryophyllene oxide, and β-selinene. Furthermore, the absence of solvents strengthens the sustainability of the whole process, as benign by design. Sampling collected during MAHD showed a progressive enrichment in monoterpenes and a decrease in sesquiterpene during the process. The volatile fraction yield and profile from MAHD were compared with those obtained from CHD, for which the oil amount was considerably lower, also having a different volatiles fingerprint.

After MAHD the residual biomass still contain most phytocannabinoids, which mainly result decarboxylated. Hence, residual hemp, unaltered from MAHD protocol, is suitable for subsequent cannabinoid recovery. Furthermore, the heating water in the biomass vessel resulted reach in polyphenols. The two main metabolites, namely luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside, were identified and quantified by means of HPLC-DAD.

In conclusion, the present investigation using a pilot scale MW reactor provided terpenes rich hydrodistillate, an enriched polyphenols fraction from the undistilled water and phytocannabinoids with a high level of decarboxylation degree.

Author contributions

The manuscript was written thanks to contributions of all the authors. All authors have given their approval to the final version of the manuscript.

CRediT authorship contribution statement

Veronika Gunjević: Investigation, Methodology. **Giorgio Grillo:** Investigation, Writing - original draft. **Diego Carnaroglio:** Conceptualization, Methodology. **Arianna Binello:** Conceptualization, Validation. **Alessandro Barge:** Formal analysis, Data curation. **Giancarlo Cravotto:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Authors warmly acknowledge the University of Turin for financial support (Ricerca locale 2020) and the agronomist Dr. Giovanni Sala (Egeria s.r.l.) for providing selected plant material.

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