IRIS AperTO

UNIVERSITÀ DEGLI STUDI DI TORINO

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1795123 since 2025-01-22T16:54:59Z

Published version:

DOI:10.1016/j.indcrop.2021.113247

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

09 March 2025

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

Veronika Gunjević[a], Giorgio Grillo[a], Diego Carnaroglio[b], Arianna Binello[a], Alessandro Barge[a], Giancarlo Cravotto[a,*]

https://doi.org/10.1016/j.indcrop.2021.113247

[a] *Dipartimento di Scienza e Tecnologia del Farmaco, University of Turin, Via P. Giuria 9, 10125, Turin, Italy*

[b] *Milestone s.r.l., Via Fatebenefratelli, 1/5, 24010 Sorisole (Bergamo), Italy.*

*Corresponding author. Tel. +39 011 670 7183; Fax: +39 011 670 7162

E-mail address: giancarlo.cravotto@unito.it

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. It is worth to point out that some concerns exist regarding the designation as "essential oil" of the extracts produced by means of this non-canonical protocol. Hence, it is possible to adopt a comprehensive term as "volatile fraction".

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.

Keywords: *Cannabis inflorescences; Terpenes; Cannabinoids; Polyphenols; Microwave-assisted hydrodistillation; Sequential extraction.*

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

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; 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.,

120 2013). Extraction rates and yields, in particular, can be increased by the enhancement of heat and
121 mass-transfer phenomena, working in synergy (Veggi et al., 2013).

122 Terpenoid yields usually vary from 0.01 to 1.5% of the inflorescence dry weight (Giese et al.,
123 2015). The hemp volatile fraction, as mentioned, consists of monoterpenes, such as α-pinene,
124 myrcene and terpinolene, and bitter-tasting sesquiterpenes, such as E-caryophyllene, α-humulene,
125 and caryophyllene oxide (Fiorini et al., 2019). These compounds can be recovered via hydro- or
126 steam distillation using Clevenger apparatus, which is the conventional extraction technique, or by
127 means of supercritical CO_2 ($scCO_2$) (Brenneisen, 2007; Markle, 2019). As abovementioned, steam
128 and hydro-distillation have numerous drawbacks and, moreover, their harsh conditions can affect
129 essential-oils quality (Markle, 2019; Lucchesi et al., 2004; Iriti et al., 2006; Ferhat et al., 2007).
130 According to literature, $scCO_2$ approaches are usually more prone to CBD and phytocannibinoids
131 recovery, due to the possibility to partially modify the selectivity of the technique by means of co-
132 solvent additions and varying the working pressure. Marzorati et al. (2020), Moreno et al. (2020).
133 Nevertheless, the main disadvantage of $scCO_2$ extraction is the fact that processing fresh plant
134 materials is impossible due to the formation of carbonic acid from CO_2 and water (Markle, 2019).
135 The required desiccation of the matrix dramatically affects the whole volatile-composition fingerprint
136 (Fiorini et al., 2019).

137 Microwave-assisted hydrodistillation (MAHD), can be an efficient alternative for *Cannabis*
138 terpenes recovery. This process is much more efficient than traditional hydro- and steam distillation
139 as the irradiation heats the plant material evenly (Markle, 2019; Ciriminna et al., 2017). Many
140 recently published studies have indicated that MW can even enhance oil extraction, by reducing
141 process time and boosting productivity, when compared with conventional extraction methods
142 (Rezvankhah et al., 2019). Abovementioned advantages of this technique opened the way to its
143 application in the extraction of phytocannabinoids, to date comprehensively reviewed (Brighenti et
144 al., 2017; Lewis-Bakker et al., 2019; Drinić et al., 2020). MAE 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. 1 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 (Figure 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 to 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 to 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.

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

220 the volatile fraction was calculated both in relation to dry matrix and on dry inflorescences. The
221 volatile fraction was then analyzed using GC-MS. The CBD and THC quantitative analysis of the
222 extract that was obtained in the optimal MAHD test was performed using UPLC-MS/MS.
223
224 2.4.2. Conventional hydrodistillation (CHD)
225 CHD was performed in order to compare the efficiency of MAHD terpene extraction to a
226 conventionally applied procedure. It was carried out according to the essential oils extraction methods
227 described in European Pharmacopoeia (2013) with few modifications due to the equipment
228 limitations. The *Cannabis* was placed in a 2 L round bottom flask and deionized water was added at
229 a solid/liquid ratio of 1 to 5. The round bottom flask was placed inside a heating mantle, whilst a
230 Clevenger-type apparatus and a refrigerant were assembled. The extraction time was 4 h. The yield
231 of the recovered hydrodistillate was expressed on dry matrix and only dry inflorescence mass. The
232 hydrodistillate yield and composition was compared with the ones obtained in MAHD tests. The
233 extraction was performed in triplicate, expressing the results as average ± S.D.
234
235 *2.5. Hydrodistillate analysis*
236
237 The GC-MS qualitative analyses of the volatile fractions obtained in MAHD and CHD were
238 performed in an Agilent Technologies 6850 Network GC System fitted with a 5973 Network Mass
239 Selective Detector, 7683B Automatic Sampler, and a capillary column Mega 5MS (length 30 m; i.d.
240 0.25 mm; film thickness 0.25 µm, Mega S.r.l., Italy) according to the method reported by Gunjević
241 et al. (2020). The identification of the individual compounds was performed with two approaches: 1)
242 by comparing the retention time e mass spectrum with standard compounds, 2) by using GC-MS
243 Wiley275 and NIST05 GC libraries from the acquired chromatograms, considering only matching

qualities 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. MAHD water

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 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 to 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)

MAHG 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 Figure 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 and 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 (2.1x50, 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 a furnace. 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 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.

3.2.Water content determination in plant material

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.

342 *3.3.Volatiles extraction*

343

344 3.3.1. Microwave-assisted hydrodistillation (MAHD)

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

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

354 First, the quantity of water added to the system to enhance the stripping power of steam was
355 screened, and its influence on the process was determined. Water addition can increase terpene yield
356 but, more importantly, it prevents the extracted material from burning (consequently, metabolites
357 degradation), thus preserving quality and use of the matrix after MAHD, such as selective
358 phytocannabinoids recovery (Markle, 2019). Moreover, material combustion during distillation can
359 lead to the release of undesired compounds into the volatile fraction. All sources of water (added and
360 contained in the plant) were heated during the extraction, generating steam that allows the release of
361 terpenes from *Cannabis* inflorescence and carries them to the distillation head. As reported in Figure
362 2A, the intermediate plant/liquid ratio of 1:1 proved to be the most efficient, as it kept the matrix wet
363 until the end of the extraction and led to the highest yield. For this reason, the remaining MAHD-
364 screening tests were carried out using this water amount. Fiorini et al. (2020) performed MAHD in a
365 similar reactor set-up, and likewise studied the water addition effect. These authors reported the
366 highest volatiles yield when 30% of water was added, assessing that higher water content caused

yield decrease. This consideration differ much from results reported in Figure 2A, according to which, when expressing the water addition in percentage, the highest volatile fraction's yield was provided 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 Figure 2B. Yields from Test 1 and Test 2 were not statistically different. However, since tap water doesn't require additional treatments as the deionized one, tap water use is preferable. 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 Figure 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 Figure 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 national regulation on psychotropic substances. According to the most recent regulation in the Italian legislation (note published from 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 represent a violation (DPR 309/90). Given the abovementioned regulations, it is mandatory to have a suitable analytical method for THC determination to verify compounds legality. 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.

CBD percent area 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. Figure 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, Figure 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 (Figure 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, Figure 4C). On the other hand, the cannabinoid area was dramatically lower, namely 0.3, 0.55, 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 volatiles yield 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.

3.3.2. Conventional hydrodistillation (CHD)

CHD 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 when 0.08 ±0.01%, w/w, 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 4h, 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, 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.

3.4.Volatile fraction analysis

3.4.1. MAHD

491 A qualitative analysis of the terpenes was performed using the GC-MS system, matching
492 93.6% of the overall composition by the comparison with standard compounds and mass spectra
493 libraries (quality ≥95%). The non-assigned compounds show very low area percentages and poor
494 libraries quality matching (<<95%). Hence, they were assumed to be barely significant. The
495 compounds contained in the sample that was obtained from the optimized MAHD Test 2 are listed
496 in Table 3, and are expressed as relative peak areas on the GC-MS chromatogram. A detailed report
497 of retention times and mass fragmentations for every detected compound is reported by Gunjević et
498 al. (2020). Whereas the relative percent area of CBD has already been reported in the paragraph
499 3.3.1., it was not shown in Table 3.

500 As can be seen from Table 3, the prevailing terpenoids with highest peak areas are as follows:
501 monoterpenes: α-pinene, β-myrcene, β-ocimene; and sesquiterpenes: E-caryophyllene, α-humulene,
502 caryophyllene oxide, and β-selinene. These are the compounds typically present in the volatile
503 hydrodistillate of European *Cannabis sativa* L. (Brenneisen, 2007). α-Pinene has a characteristic pine
504 fragrance and exhibits antiseptic properties. β-Myrcene is characterized by a musky fragrance as well
505 as antioxidant and chemo-protective effects. Caryophyllene has a peppery fragrance, and gastro-
506 protective and anti-inflammatory biological activity (Leghissa et al., 2018). Moreover, it is a Food
507 and Drug Administration (FDA) approved food additive. Caryophyllene oxide is used as the marker
508 compound for marijuana detection by trained dogs (Fiorini et al., 2019).

509 Fiorini et al. (2020) performed MAHD of *Cannabis* volatiles, obtaining a CBD enriched
510 volatile fraction. The main components present in this extract were caryophyllene, CBD, α-
511 humulene, α-pinene, caryophyllene oxide and myrcene. Therefore, the recoevered terpenes
512 composition is similar to Test 13 extract, even if composition deviations are observed.

513 As already mentioned, the extraction that gave the highest hydrodistillate yield (Test 2) was
514 repeated in order to observe how the composition profile evolves during extraction (Test 13). The
515 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 Figure 5) at each sampling time. For the sake of comparison, percentage areas were normalized exclusively in relation to the abovementioned compounds.

On Figure 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 Figure 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.

3.4.2. 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 3.3.2., 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. 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 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.

3.5.MAHD water – 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

565 of green extraction (Chemat et al., 2019). Polyphenols are some of today's most interesting
566 phytochemicals. They possess several biological effects, including antioxidant, antibacterial, anti-
567 inflammatory, and chemo-preventive power (Cravotto et al., 2018). The MW-assisted extraction of
568 hemp polyphenols has been described in several papers. Teh et al. (2014) have investigated the use
569 of MW as a prior step to US-assisted polyphenol extraction from defatted hemp seed cake. The results
570 have shown that the irradiation of this residue can enhance the metabolites extraction, maximizing
571 the polyphenols yield. Drinić et al. (2020) unified the aforementioned approaches, developing an
572 optimization of hemp MAE for phenols, flavonoids, and phytocannabinoids in ethanol. In this case,
573 MAE was found to be a simple, fast, and efficient extraction method for the cited classes of
574 metabolites, preserving at the same time the high antioxidant activity of the extract.

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

579

580 3.5.1. Polyphenol enrichment and HPLC analysis

581 The SPE was used to purify polyphenolic water fraction obtained from optimized MAHD for
582 the sake of analysis. The concentrated polyphenol fraction yield reached 10.31 ±0.45%, w/w,
583 calculated in relation to the dry raw extract. The process led to an overall TPC content of 51.71
584 ±2.25%, w/w, calculated in relation to the dry purified sample, achieving a nearly 10-fold metabolite
585 concentration compared with the raw dry extract.

586 Literature suggests that the main polyphenols in *Cannabis sativa* L. are flavonoids (Koltai
587 and Namdar, 2020; Nagy et al., 2019). Nevertheless, phenolic acids have also been detected in
588 *Cannabis* plant (Izzo et al., 2020). Therefore, HPLC-DAD was used to analyse polyphenols
589 belonging to the aforesaid classes present in the enriched sample. Two main peaks were identified,

590 on the basis of standard compounds, as flavone products, namely luteolin-7-O-glucoside and
591 apigenin-7-O-glucoside, with an amount of 2.84 ±0.12%, w/w and 2.58 ±0.11%, w/w, respectively,
592 when calculated in relation to the dry purified water fraction. Moreover, the absorption spectrum of
593 each compound detected was thoroughly revised. In particular, six signals, besides luteolin and
594 apigenin glucosides, were detected at 340 nm and featured a spectrum that is characteristic for
595 flavones, as reported in Gunjević et al. (2020). However, the lack of specific standards means that
596 identification and quantification were impossible. In agreement with previous investigations the main
597 polyphenols in low-THC *Cannabis* cultivars were flavones (Brenneisen, 2007).

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

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

606

607 *3.6. Cannabinoid MAHG extraction*

608

609 Reflux is the conventional extraction method to achieve cannabinoids recovery from
610 *Cannabis* inflorescence (Baranauskaite et al., 2020; De Vita et al., 2020). It entails a long extraction
611 time and ineffective conductive heating. Moreover, it requires ethanol, a widely used but potentially
612 flammable solvent. A preliminary MAHG test was performed to evaluate the possibility of
613 overcoming the disadvantages of the classical extraction approach. Steam was introduced into the
614 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 yield of hydrodistilled oil was 0.35 ±0.02% w/w, expressed in relation to dry inflorescence mass. The extract 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 only 0.12 ±0.01%, w/w in relation to dry inflorescence, also having a different volatiles fingerprint.

After MAHD the residual biomass still contain most phytocannabinoids, which mainly result decarboxylated (69.01 ±0.98% for THC and 74.32 ±1.02% for CBD). 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 (5.35 ±0.23%, w/w in the dry extract). 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 information

Corresponding Author

* E-mail: giancarlo.cravotto@unito.it

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.

Acknowledgements

The authors warmly acknowledge Milestone s.r.l. for providing the ETHOS X MW reactor and their assistance as well as the agronomist Dr. Giovanni Sala (Egeria s.r.l.) for providing selected plant material.

References

Aizpurua-Olaizola, O., Soydaner, U., Öztürk, E., Schibano, D., Simsir, Y., Navarro, P., Etxebarria, N., Usobiaga, A., 2016. Evolution of the Cannabinoid and Terpene Content during the Growth of Cannabis sativa Plants from Different Chemotypes. J. Nat. Prod. 79, 324–331. https://doi.org/10.1021/acs.jnatprod.5b00949

Baranauskaite, J., Marksa, M., Ivanauskas, L., Vitkevicius, K., Liaudanskas, M., Skyrius, V., Baranauskas, A., 2020. Development of extraction technique and GC/FID method for the analysis of cannabinoids in Cannabis sativa L. spp. santicha (hemp). Phytochem. Anal. 31, 516–521. https://doi.org/10.1002/pca.2915

BDS Analytics, 2019. The Global Cannabinoids Market: Will CBD Overtake THC? | White Paper [WWW Document]. URL https://bdsa.com/will-cbd-overtake-thc-white-paper/ (accessed 11.20.20).

Booth, J.K., Page, J.E., Bohlmann, J., 2017. Terpene synthases from Cannabis sativa. PLoS One 12, 1–20. https://doi.org/10.1371/journal.pone.0173911

Brenneisen, R., 2007. Chemistry and Analysis of Phytocannabinoids and Other Cannabis Constituents. Marijuana and the Cannabinoids 17–49. https://doi.org/10.1007/978-1-59259-947-9_2

Brighenti, V., Pellati, F., Steinbach, M., Maran, D., Benvenuti, S., 2017. Development of a new extraction technique and HPLC method for the analysis of non-psychoactive cannabinoids in fibre-type Cannabis sativa L. (hemp). J. Pharm. Biomed. Anal. 143, 228–236. https://doi.org/10.1016/j.jpba.2017.05.049

Chemat, F., Abert-Vian, M., Fabiano-Tixier, A.S., Strube, J., Uhlenbrock, L., Gunjevic, V., Cravotto, G., 2019. Green extraction of natural products. Origins, current status, and future challenges. TrAC - Trends Anal. Chem. 118, 248–263. https://doi.org/10.1016/j.trac.2019.05.037

Chemat, F., Vian, M.A., Cravotto, G., 2012. Green extraction of natural products: Concept and principles. Int. J. Mol. Sci. 13, 8615–8627. https://doi.org/10.3390/ijms13078615

Ciriminna, R., Fidalgo, A., Delisi, R., Carnaroglio, D., Grillo, G., Cravotto, G., Tamburino, A., Ilharco, L.M., Pagliaro, M., 2017. High-Quality Essential Oils Extracted by an Eco-Friendly Process from Different Citrus Fruits and Fruit Regions. ACS Sustain. Chem. Eng. 5, 5578–5587. https://doi.org/10.1021/acssuschemeng.7b01046

Council of Europe, 2013. European Pharmacopeia 7.0, 8th ed, European Pharmacopeia. Srrasbourg.

Cravotto, G., Mariatti, F., Gunjevic, V., Secondo, M., Villa, M., Parolin, J., Cavaglià, G., 2018. Pilot scale cavitational reactors and other enabling technologies to design the industrial recovery of polyphenols from agro-food by-products, a technical and economical overview. Foods 7, 1–14. https://doi.org/10.3390/foods7090130

De Vita, D., Madia, V.N., Tudino, V., Saccoliti, F., De Leo, A., Messore, A., Roscilli, P., Botto, A., Pindinello, I., Santilli, G., Scipione, L., Costi, R., Di Santo, R., 2020. Comparison of different methods for the extraction of cannabinoids from cannabis. Nat. Prod. Res. 34, 2952–2958. https://doi.org/10.1080/14786419.2019.1601194

De Vita, D., Madia, V.N., Tudino, V., Saccoliti, F., De Leo, A., Messore, A., Roscilli, P., Botto, A., Pindinello, I., Santilli, G., Scipione, L., Costi, R., Di Santo, R., 2019. Comparison of different methods for the extraction of cannabinoids from cannabis. Nat. Prod. Res. 0, 1–7. https://doi.org/10.1080/14786419.2019.1601194

Drinić, Z., Vladić, J., Koren, A., Zeremski, T., Stojanov, N., Kiprovski, B., Vidović, S., 2020. Microwave-assisted extraction of cannabinoids and antioxidants from Cannabis sativa aerial parts and process modeling. J. Chem. Technol. Biotechnol. 95, 831–839. https://doi.org/10.1002/jctb.6273

Elzinga, S., Fischedick, J., Podkolinski, R., Raber, J., 2015. Cannabinoids and Terpenes as Chemotaxonomic Markers in Cannabis. Nat. Prod. Chem. Res. 03.

https://doi.org/10.4172/2329-6836.1000181

Fathordoobady, F., Singh, A., Kitts, D.D., Pratap Singh, A., 2019. Hemp (Cannabis Sativa L.) Extract: Anti-Microbial Properties, Methods of Extraction, and Potential Oral Delivery. Food Rev. Int. 35, 664–684. https://doi.org/10.1080/87559129.2019.1600539

Ferhat, M., Meklati, B.Y., Chemat, F., 2007. Comparison of different isolation methods of essential oil from Citrus fruits: cold pressing, hydrodistillation and microwave 'dry' distillation. FLAVOUR Fragr. J. 22, 494–504. https://doi.org/10.1002/ffj

Fiorini, D., Molle, A., Nabissi, M., Santini, G., Benelli, G., Maggi, F., 2019. Valorizing industrial hemp (Cannabis sativa L.) by-products: Cannabidiol enrichment in the inflorescence essential oil optimizing sample pre-treatment prior to distillation. Ind. Crops Prod. 128, 581–589. https://doi.org/10.1016/j.indcrop.2018.10.045

Fiorini, D., Scortichini, S., Bonacucina, G., Greco, N.G., Mazzara, E., Petrelli, R., Torresi, J., Maggi, F., Cespi, M., 2020. Cannabidiol-enriched hemp essential oil obtained by an optimized microwave-assisted extraction using a central composite design. Ind. Crops Prod. 154, 112688. https://doi.org/10.1016/j.indcrop.2020.112688

Giese, M.W., Lewis, M.A., Giese, L., Smith, K.M., 2015. Development and validation of a reliable and robust method for the analysis of cannabinoids and terpenes in cannabis. J. AOAC Int. 98, 1503–1522. https://doi.org/10.5740/jaoacint.15-116

Graf, B.A., Milbury, P.E., Blumberg, J.B., 2005. Flavonols, flavones, flavanones, and human health: Epidemiological evidence. J. Med. Food 8, 281–290. https://doi.org/10.1089/jmf.2005.8.281

Gulluni, N., Re, T., Loiacono, I., Lanzo, G., Gori, L., MacChi, C., Epifani, F., Bragazzi, N., Firenzuoli, F., 2018. Cannabis Essential Oil: A Preliminary Study for the Evaluation of the Brain Effects. Evidence-based Complement. Altern. Med. 2018. https://doi.org/10.1155/2018/1709182

Gunjević, V., Grillo, G., Carnaroglio, D., Binello, A., Barge, Alessandro Cravotto, G., 2020. Analytical dataset of terpenes, cannabinoids and polyphenols form Cannabis sativa L. by pilot-scale microwave-assisted extraction. Data Br. submitted.

GVR, 2019. Global Polyphenols Market Size & Share | Industry Report, 2019-2025 [WWW Document]. URL https://www.grandviewresearch.com/industry-analysis/polyphenols-market-analysis (accessed 11.20.20).

Hillis, W.E., Swain, T., 1959. The phenolic constituents of Prunus domestica. II.—The analysis of tissues of the Victoria plum tree. J. Sci. Food Agric. 10, 135–144. https://doi.org/10.1002/jsfa.2740100211

Iriti, M., Colnaghi, G., Chemat, F., Smadja, J., Faoro, F., Visinoni, F.A., 2006. Histo-cytochemistry and scanning electron microscopy of lavander glandular trichomes following conventional and microwave-assisted hydrodistillation of essential oils: A comparative study. Flavour Fragr. J. 21, 704–712. https://doi.org/10.1002/ffj.1692

Izzo, L., Castaldo, L., Narváez, A., Graziani, G., Gaspari, A., Rodríguez-Carrasco, Yelko Ritieni, A., 2020. Analysis of Phenolic Compounds in Commercial Cannabis sativa L. Inflorescences Using UHPLC-Q-Orbitrap HRMS. Molecules 25, 631.

Jiang, N., Doseff, A.I., Grotewold, E., 2016. Flavones: From biosynthesis to health benefits. Plants 5, 1–1256. https://doi.org/10.3390/plants5020027

Koltai, H., Namdar, D., 2020. Cannabis Phytomolecule “Entourage”: From Domestication to Medical Use. Trends Plant Sci. 25, 976–984. https://doi.org/10.1016/j.tplants.2020.04.007

Leghissa, A., Hildenbrand, Z.L., Schug, K.A., 2018. A review of methods for the chemical characterization of cannabis natural products. J. Sep. Sci. 41, 398–415. https://doi.org/10.1002/jssc.201701003

Lewis-Bakker, M.M., Yang, Y., Vyawahare, R., Kotra, L.P., 2019. Extractions of Medical Cannabis Cultivars and the Role of Decarboxylation in Optimal Receptor Responses.

Cannabis Cannabinoid Res. 4, 183–194. https://doi.org/10.1089/can.2018.0067

Lucchesi, M.E., Chemat, F., Smadja, J., 2004. Solvent-free microwave extraction of essential oil from aromatic herbs: Comparison with conventional hydro-distillation. J. Chromatogr. A 1043, 323–327. https://doi.org/10.1016/j.chroma.2004.05.083

Markle, S., 2019. Strain-Specifi c Isolation of Terpenes Utilizing Microwave-Assisted Extraction. Cannabis Sci. Technol. 2, 50–76.

Marzorati, S., Friscione, D., Picchi, E., Verotta, L., 2020. Cannabidiol from inflorescences of Cannabis sativa L.: Green extraction and purification processes. Ind. Crops Prod. 155, 112816. https://doi.org/10.1016/j.indcrop.2020.112816

Matešić, N., Jurina, T., Benković, M., Panić, M., Valinger, D., Gajdoš Kljusurić, J., Jurinjak Tušek, A., 2020. Microwave-assisted extraction of phenolic compounds from Cannabis sativa L.: optimization and kinetics study. Sep. Sci. Technol. 00, 1–14. https://doi.org/10.1080/01496395.2020.1804938

McAllister, S.D., Soroceanu, L., Desprez, P.Y., 2015. The Antitumor Activity of Plant-Derived Non-Psychoactive Cannabinoids. J. Neuroimmune Pharmacol. 10, 255–267. https://doi.org/10.1007/s11481-015-9608-y

Mcgraw, G.W., Hemingway, R.W., Ingram, L.L., Canady, C.S., Mcgraw, W.B., 1999. Thermal degradation of terpenes: Camphene, Δ3-carene, limonene, and α-terpinene. Environ. Sci. Technol. 33, 4029–4033. https://doi.org/10.1021/es9810641

Moreno, T., Montanes, F., Tallon, S.J., Fenton, T., King, J.W., 2020. Extraction of cannabinoids from hemp (Cannabis sativa L.) using high pressure solvents: An overview of different processing options. J. Supercrit. Fluids 161, 104850. https://doi.org/10.1016/j.supflu.2020.104850

Nagy, D.U., Cianfaglione, K., Maggi, F., Sut, S., Dall'Acqua, S., 2019. Chemical Characterization of Leaves, Male and Female Flowers from Spontaneous Cannabis (Cannabis sativa L.)

Growing in Hungary. Chem. Biodivers. 16. https://doi.org/10.1002/cbdv.201800562

Namdar, D., Mazuz, M., Ion, A., Koltai, H., 2018. Variation in the compositions of cannabinoid and terpenoids in Cannabis sativa derived from inflorescence position along the stem and extraction methods. Ind. Crops Prod. 113, 376–382. https://doi.org/10.1016/j.indcrop.2018.01.060

Rezvankhah, A., Emam-Djomeh, Z., Safari, M., Askari, G., Salami, M., 2019. Microwave-assisted extraction of hempseed oil: studying and comparing of fatty acid composition, antioxidant activity, physiochemical and thermal properties with Soxhlet extraction. J. Food Sci. Technol. 56, 4198–4210. https://doi.org/10.1007/s13197-019-03890-8

Teh, S.S., Niven, B.E., Bekhit, A.E.D.A., Carne, A., Birch, E.J., 2014. The Use of Microwave and Pulsed Electric Field as a Pretreatment Step in Ultrasonic Extraction of Polyphenols from Defatted Hemp Seed Cake (Cannabis sativa) Using Response Surface Methodology. Food Bioprocess Technol. 7, 3064–3076. https://doi.org/10.1007/s11947-014-1313-y

Veggi, P.C., Martinez, J., Meireles, M.A. a, 2013. Microwave-assisted Extraction for Bioactive Compounds, Microwave-assisted Extraction for Bioactive Compounds: Theory and Practice. Springer, Boston, MA. https://doi.org/10.1007/978-1-4614-4830-3

Wang, M., Wang, Y.H., Avula, B., Radwan, M.M., Wanas, A.S., Van Antwerp, J., Parcher, J.F., Elsohly, M.A., Khan, I.A., 2016. Decarboxylation Study of Acidic Cannabinoids: A Novel Approach Using Ultra-High-Performance Supercritical Fluid Chromatography/Photodiode Array-Mass Spectrometry. Cannabis Cannabinoid Res. 1, 262–271. https://doi.org/10.1089/can.2016.0020

Yang, Y., Lewis, M.M., Bello, A.M., Wasilewski, E., Clarke, H.A., Kotra, L.P., 2017. Cannabis sativa (Hemp) Seeds, Δ 9 -Tetrahydrocannabinol, and Potential Overdose . Cannabis Cannabinoid Res. 2, 274–281. https://doi.org/10.1089/can.2017.0040

Zuiderweg, F.J., Harmens, A., 1958. The influence of surface phenomena on the performance of

distillation columns. Chem. Eng. Sci. 9, 89–103. https://doi.org/10.1016/0009-2509(58)80001-9

Tables

Table 1. *Cannabis* MAHD 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 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 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[c]	2.69	Deionized	1/1	-	-	-	-	-	-

[a] yields expressed on dry matter

[b] GC-MS relative area

[c] no results reported since this extraction was performed to evaluate the composition of the volatile fraction during the extraction by periodical sampling

Table 2. THC and CBD 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.

Table 3. Terpene fraction profile obtained in MAHD 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%).

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

Table 5. Volatile fraction profile obtained in CHD. 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%).

Figures:

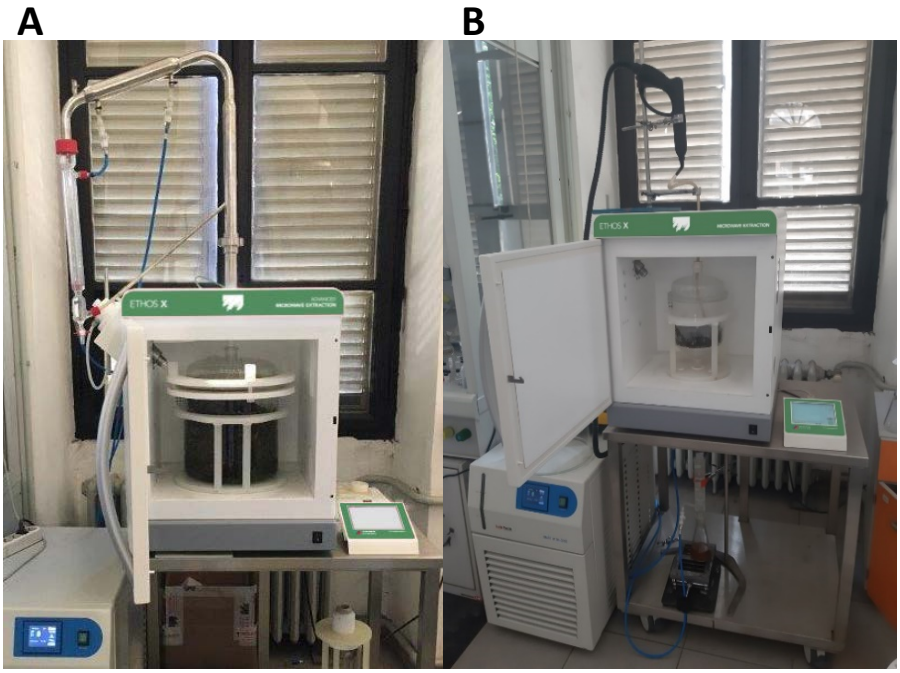

Figure 1. ETHOS X: A) MAHD set-up, B) MAHG set-up.

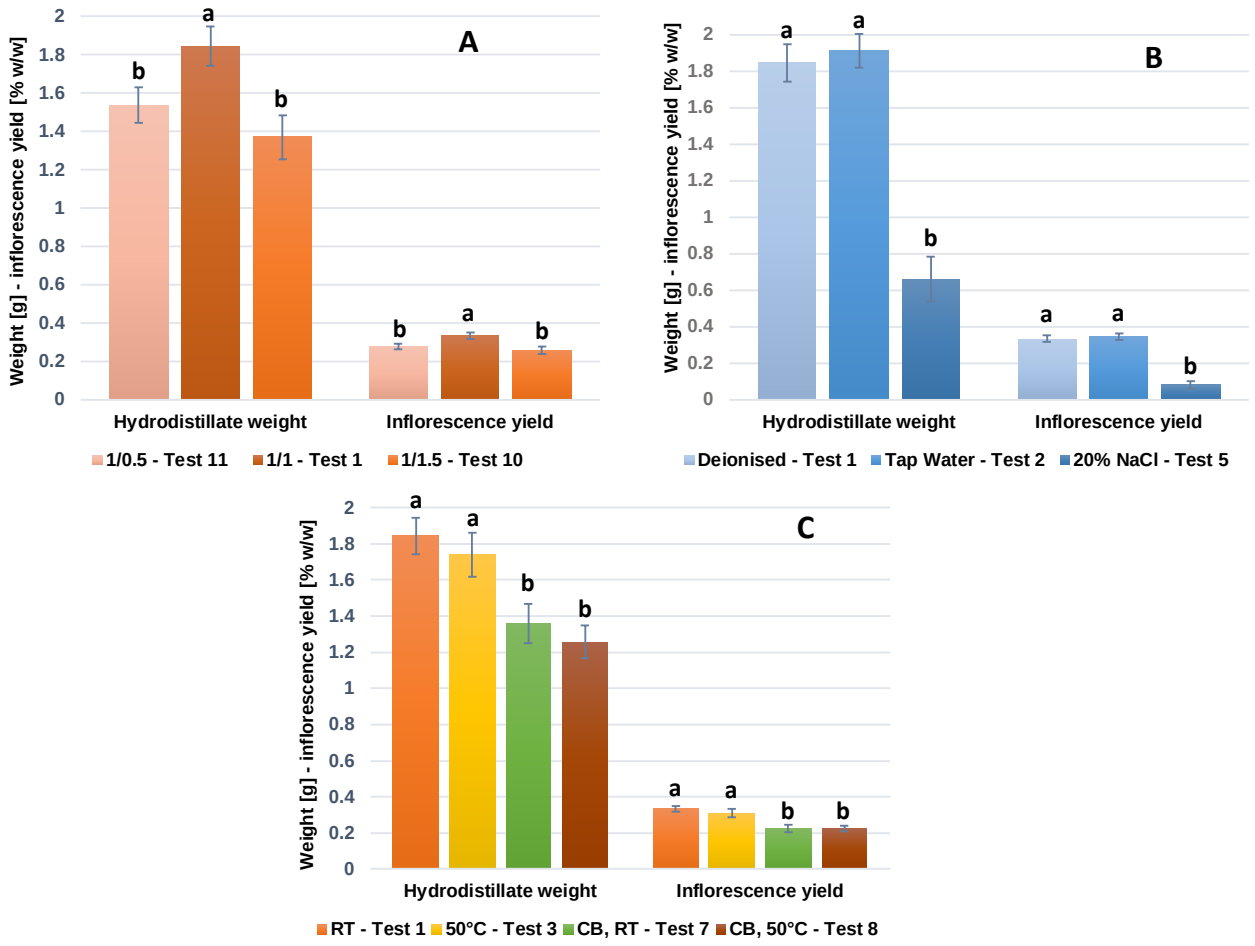

Figure 2. MAHD hydrodistillate yield and mass trend with different water feeds: A) plant/liquid ratio w/w:1/0.5, 1/1, 1/1.5; B) deionized, tap water, 20% NaCl; C) RT *vs.* hot-water (50°C) addition. Data on hydrodistillate weight (g) and only inflorescence yield (%, w/w). Results are expressed as average values ± S.D.. Presented values followed by different lower-case letters (a–b) are significantly different from each other ($p < 0.05$) according to water feed used, as determined by Tukey's HSD test.

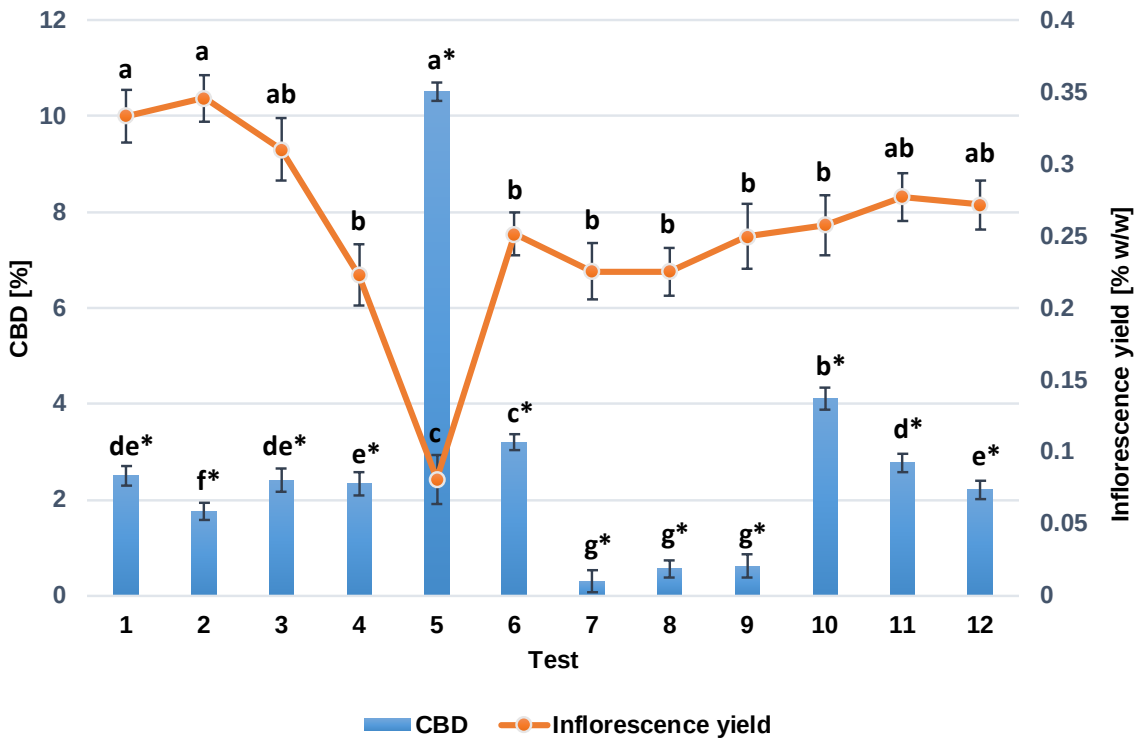

Figure 3. CBD trend for MAHD and hydrodistillate yield expressed on dry inflorescence: general outlook. Data of CBD in all performed tests are reported as percent area of GC-MS chromatograms. Results are expressed as average values ± S.D.. Values that are statistically different from each other ($p < 0.05$) are indicated with lower-case letters (a-c; a*- g*), as determined by Tukey's HSD test.

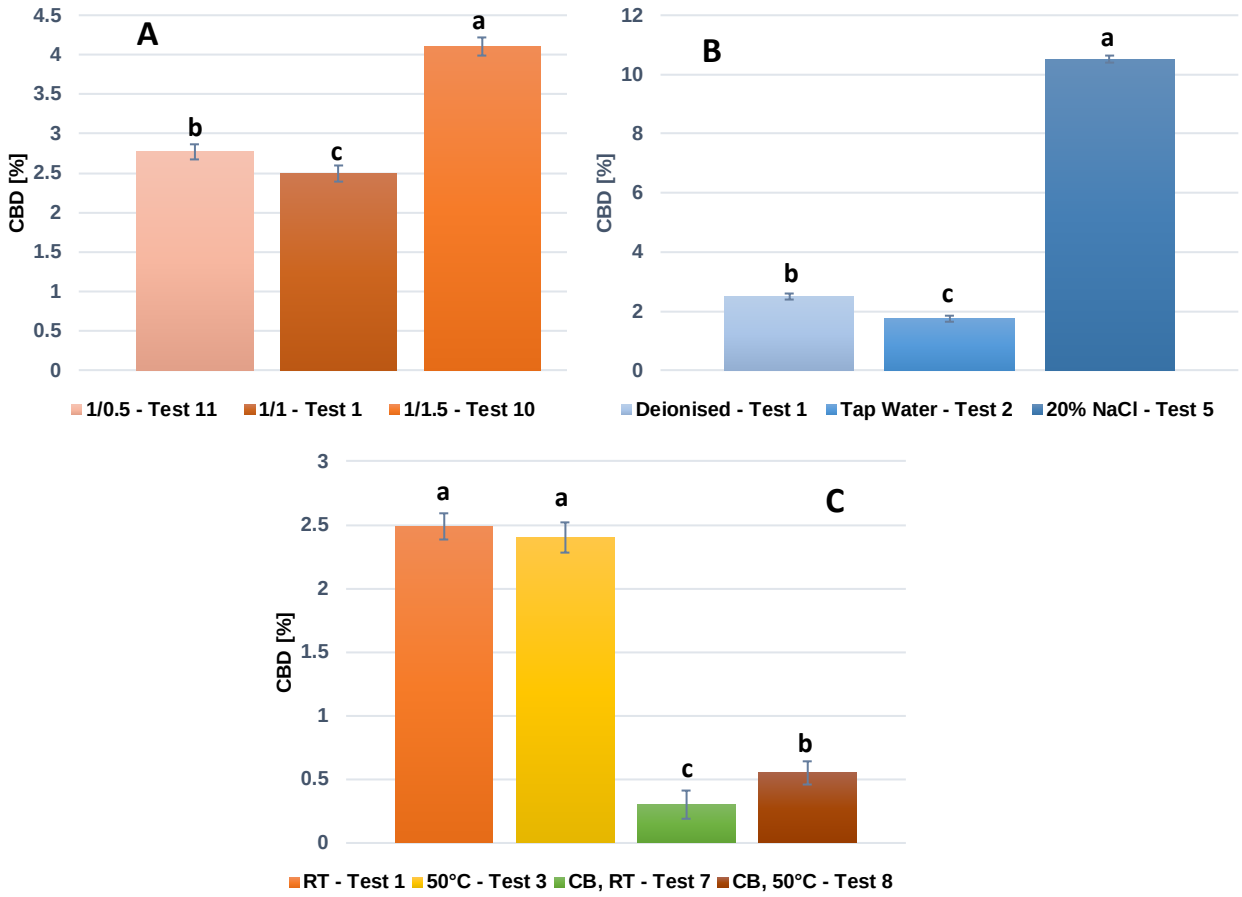

Figure 4. CBD trend, expressed as relative GC-MS percent area, for MAHD. A) water/matrix ratio. B) Type of water feed. C) RT/hot water (50°C), CB application. Results are expressed as average values ± S.D. Presented values followed by different lower-case letters (a–c) are significantly different from each other ($p < 0.05$) according to extraction water feed used, as determined by Tukey's HSD test.

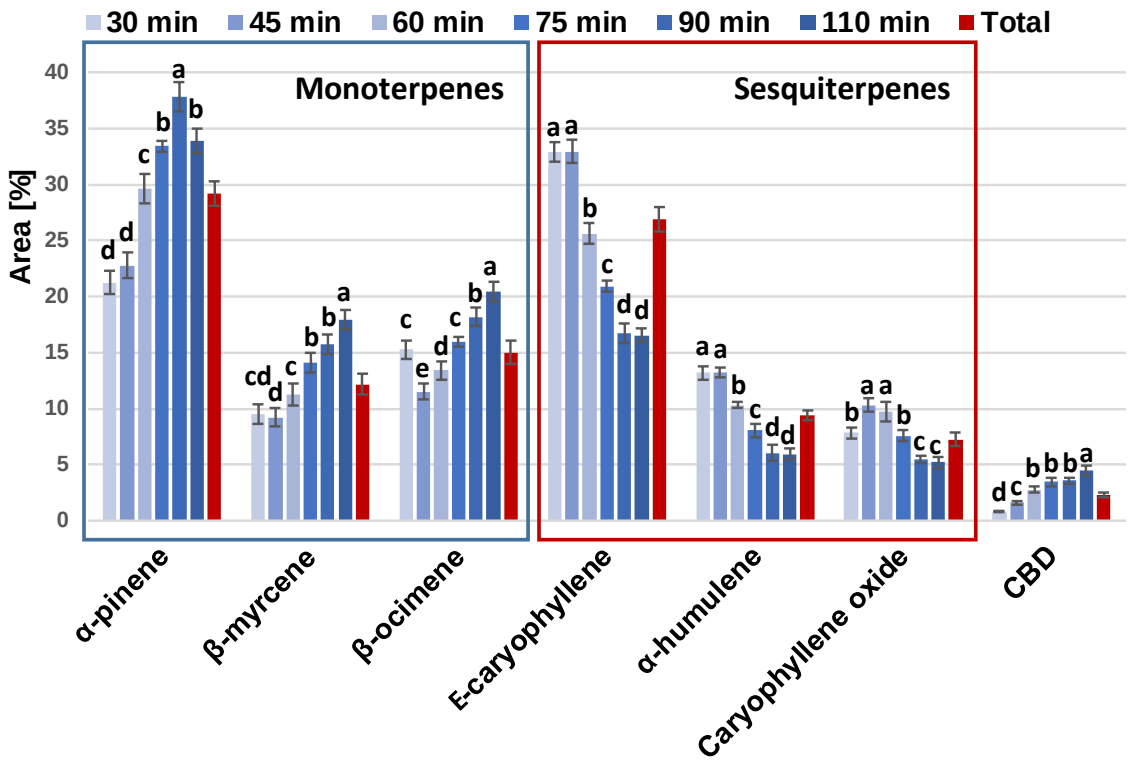

Figure 5. Test 13 MAHD sampling: main terpenes composition distribution. CBD relative area variation in time. Both main terpenes and CBD are expressed as relative peak areas obtained by GC-MS quantification. Results are expressed as average values ± SD. Presented values followed by different lower-case letters (a–d) are significantly different from each other ($p < 0.05$) according to extraction water feed used, as determined by Tukey's HSD test. Statistical analysis of the total united samples was not depicted on this Figure, since herein comparison of specific samples was performed.