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Cannabidiol from inflorescences of *Cannabis sativa* L.: Green extraction and purification processes

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ABSTRACT

This work investigates an extraction and purification downstream process able to provide cannabidiol (CBD) enriched products starting from inflorescences of *Cannabis sativa* L. Even though the legislation concerning non-psychoactive Cannabis derivatives is still an open issue, the interest in this compound is justified worldwide by the scientifically demonstrated health beneficial effects of CBD for the treatment of many disorders. For these reasons, the interest in CBD-based formulations, for the sake of readiness for the future market demands, is quite high. Despite the fact that the recent literature is highly addressed to the topic of cannabinoids and their related biological activities, there is still a general lack in strategic proposals aiming at recovering and purifying specifically CBD through scalable processes.

In this work, preliminary studies were addressed to convert the main product of the plant metabolism, cannabidiolic acid, to CBD by heat-treatment-induced decarboxylation of the dried biomass. Then, a "green strategy", supercritical CO_2 extraction, enabled to yield a 50 % w/w CBD-enriched oil, avoiding any use of toxic organic solvents. Yields and compositions of methanolic extraction and supercritical CO_2 were compared. Results confirmed the enhanced selectivity of supercritical CO_2. A winterization process, followed by flash chromatography, successfully removed waxes and the psychotropic fraction, providing an almost 80 % w/w CBD-enriched final product.

1. Introduction

Cannabis sativa L. (hemp) plants have been used all over the world since ancient times due to their multifunctional characteristics, whose most important derived products are: i) oil and proteins, used in food and feed production, ii) fibers, used in the paper and textile industry and iii) resins, displaying many important biological/pharmacological activities in humans (Brown, 1998). As a ubiquitous and well-adaptable plant even in different climates, Cannabis accompanied the history of traditional medicine for millennia, increasing the awareness that certain plants were better for fiber, others for edible seed, while others were pharmacologically superior. The isolation and structural elucidation of unique compounds, namely cannabinoids, was finalized in the 19th century to justify its wide use as a medicinal plant (Mechoulam and Gaoni, 1967). Even if the cannabis plant contains more than one hundred of individual cannabinoids, the predominant compounds produced by the secondary metabolism are delta-9-tetrahydrocannabinolic acid (Δ^9-THCA) and cannabidiolic acid (CBDA). Both of them, undergoing decarboxylation, are converted into the mostly famous and discussed cannabinoids: delta-9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) (Sirikantaramas and Taura, 2017). Studies have revealed that these compounds are able to interact with the receptors of the endocannabinoid system, giving a feedback response able to regulate humor, pain, muscle relaxation, hunger, nausea *etc.* (Hacke et al., 2019; Klumpers and Thacker, 2019). The main point that still is the center of many legislative debates is related to the psychoactive fraction, due to the presence of Δ^9-THC, responsible, upon administration, of side effects such as dysphoria, loss of judging capacity, panic, allucinations, *etc.* (Reyes et al., 1973). The Italian legislative framework (law 242/2016), following the European one, has compelled farmers to grow plants characterized by high levels of CBDA (and hence CBD) by gene selection, able to respect the limit established by law (Cas et al.,

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2019). However, there is still much discontinuity and unclearness and law threshold limits are subjected to changes because, apart from the recreational use, the pharmacological interest in cannabinoids and their derived products is increasing.

This frame might be responsible of the incompleteness of full strategies and protocols aiming at isolating specific compounds for their further large scale production. The pharmacological interest in the non-psychotropic fraction, in particular concerning cannabidiol, is of high impact due to its activity in the central and peripheral nervous system. In the absence of THC, CBD was demonstrated to be still effective in the treatment of diseases like Alzheimer, multiple sclerosis, epilepsy and depression or anxiety due to its neuroprotective and anticonvulsant effects (Mechoulam et al., 2007). Cannabidiol has also been demonstrated to be effective in the treatment of pain and useful in dermatological field (Costa et al., 2007; Hammell et al., 2016; Wilkinson and Williamson, 2007). Apart from these health-related applications, CBD has been studied also for its potential and promising insecticidal activity (Benelli et al., 2018; Park et al., 2019).

Typing "cannabidiol" in Scifinder® and filtering results just for the last 20 years, it is interesting to notice that most of the work, about 60 %, are centered on *in vitro* and *in vivo* pharmacological activity of the compound; noteworthy, 30 % are still investigating and developing analytical methods able to discriminate the presence of other cannabinoids. Finally, only 6% of the published researches is interested in the extraction protocols. However, even this small portion of the literature seems more interested in "total extracts", by means of different extraction and analytical methodologies, still lacking in strategies able to target a specific and highly valuable compound like CBD (Bouayoun et al., 2018; Da Porto et al., 2014; Perrotin-Brunel, 2011; Rovetto and Aieta, 2017).

According to global trends, industries are always looking for "green" products and technologies, able to replace conventional ones. This work presents as core strategy, a supercritical CO_2 extraction methodology, characterized by low environmental impacts: organic solvents are avoided and carbon dioxide is used as the extraction fluid, ensuring safe and selective extraction strategy, with the possibility to recycle the employed CO_2 in industrial plants (De Melo et al., 2014). A further cascade process strategy, comprising purification protocols has been employed, able to produce a cannabidiol-enriched final product.

2. Materials and methods

2.1. Chemicals

The hemp cultivar Finola was grown in Italy and its inflorescences were provided as dried biomass by Azienda Agricola Colornese di Sandei Maurizio Battista (Colorno, Parma, Italy).

All chemicals were used without further treatment. Methanol, ethanol and water, when used for liquid chromatography, were purchased from Sigma-Aldrich as ultra performance liquid chromatography-grade. Cannabidiol 1.0 mg mL^{-1} methanolic standard solution (certified reference material, Cerilliant®) and Δ^9-THC-D_3, delta-9-tetrahydrocannabinol 0.1 mg mL^{-1} methanolic standard solution were purchased from Sigma-Aldrich. A carbon dioxide tube (CO_2 purity 3.5) was purchased from Sapio, Italy.

2.2. Milling

The dried *Cannabis sativa* L. inflorescences were milled (about 50 μm particle size) using a knife mill at its maximum velocity for 10 s. To avoid powder heating during blending and the consequent degradation of thermolabile and volatile species, liquid nitrogen was added during the milling procedure. The pulverized sample was stored in the dark at ambient conditions.

2.3. Heat-treatment

Heat treatment was performed on pulverized dried inflorescences using an oven under static air. The powder was introduced in the cold oven on an inert tray and then the temperature ramp was set at 1 °C min^{-1} until the set temperature of 100 °C was reached and maintained for the desired time.

2.4. Conventional methanolic extraction

The pulverized sample (4.5058 g) was extracted in 45 mL of methanol for 2 h stirring at room temperature. The suspension was filtrated on a Buchner funnel and organic solvent was evaporated under *vacuum* by rotavapor (Buchi R210). The extract was analysed by liquid chromatography.

2.5. Supercritical CO_2 extraction

Supercritical CO_2 extractions (sc-CO_2) were performed using a pilot unit SFT110XW System (Supercritical Fluid Technologies, Inc., USA). It consists of an SFT10 CO_2 pump with a Peltier Cooler, a 100 cm^3 stainless steel extractor vessel inserted in an oven and a collection vial. For each experiment, almost 18 g of pulverized dried hemp inflorescences were loaded in the extraction vessel. After a literature overview and some optimization trials, the operative pressure was set as 380 bar in all experiments, while the temperature of the vessel and the temperature of the restrictor block were maintained at 60 °C and 80 °C, respectively, throughout the extraction period (Attard et al., 2018; Rovetto and Aieta, 2017). For each extraction experiment eight cycles, comprising 10 min of maceration time in static conditions and 10 min of dynamic conditions, were performed. In dynamic conditions, the valves were opened and the extract was collected in a vial, keeping a CO_2 gas constant flow rate of 0.28 SCMH (standard cubic meter per hour). The extracts were then stored in a freezer for subsequent analysis.

2.6. Ultra performance liquid chromatography coupled to mass spectrometry analysis

The extract composition was analyzed with Waters ACQUITY UPLC (Ultra performance liquid chromatography) system (Waters corp., United States) equipped with a quaternary solvent manager system, autosampler, thermostated column compartment and a dual-wavelength UV–vis detector. The analytical separation was performed using an ACQUITY UPLC® BEH C18 column (1.7 μm, 2.1 × 50 mm). The mobile phase was a mixture of water (+ 0.1 % v/v formic acid):methanol = 20:80 at a flow rate of 0.2 mL min^{-1} in isocratic mode. Methanolic solutions of samples were filtered (0.2 μm nylon filters) and injected (2 μl) in the system. The column temperature was maintained at 35 °C and the wavelength was set at 225 nm. Data were processed with Empower 3 workstations.

When necessary, chromatographic separation was followed by a mass spectrometry (LCQ Fleet Thermofisher) analysis. A positive electrospray mode was used for the ionization of molecules with a capillary voltage of 10 V, at a capillary temperature of 275 °C. The heater temperature was set at 150 °C, the gas flow rate was 20 (arb) and the spray voltage was 5.0 kV. The monitored mass range was *m/z* 100–800.

2.7. Winterization

For each gram of extract obtained from sc-CO_2, 10 mL of ethanol were added. The solution/suspension was warmed up to 40 °C for 10 min while stirring and then frozen −15 °C for 36 h. The solid-liquid mixture was then cold-filtered, the solvent was evaporated from the fil-

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tered solution by rotary evaporator and the residue was dried under vacuum pump for 4 h.

2.8. Flash chromatography equipment

The purification was performed on a CombiFlash system (Nextgen 300+, Teledyne Isco Lincoln, USA) on a RediSep C18 column. 0.7 g of winterized sample were dissolved in the smallest volume of methanol (*ca.* 3.5 mL). The sample was pre-adsorbed on 2 g of Celite or C18 silica powder and then dried under vacuum. The composite was loaded in the sample holder before the separation column. The chromatographic separation was performed at a flow rate of 30 mL min^{-1} using methanol and water (+0.1 % v/v formic acid) following the method: 0 min: 60 % MeOH, 15 min 100 % MeOH, 16–20 min: 60 % MeOH. The signal was monitored at 225 nm. The solvent was evaporated by rotary evaporator from the fractions of interest and the collected sample was dried under vacuum pump for at least 4 h.

2.9. Sequential downstream extraction/purification process

The proposed experimental protocol, able to yield the final CBD-enriched product, included the following steps: *1*) the dried inflorescences were pulverized by means of a knife mill in order to get a higher surface-to-volume ratio; *2*) the powder underwent heat treatment for 6 h at 100 °C under static air, in order to maximize the kinetics of decarboxylation of CBDA to CBD, without any adverse effect on the other side reactions occurring; *3*) the vessel of the supercritical CO_2 extractor was loaded with the biomass deriving from step 2. Temperature and pressure were set at 60 °C and 380 bar, respectively, and the extraction was conducted alternating static (maceration) and dynamic (collection) cycles. After 2 h and 40 min, the extraction was interrupted because there was no further significant enhancement of the extracted mass; *4*) a winterization procedure was then carried out using ethanol, aiming at precipitating waxes; *5*) flash chromatography was finally employed for a further purification, able to separate the undesired compounds from the target one.

3. Results and discussion

3.1. Cannabidiol and delta-9-tetrahydrocannabinol calibration lines

Six dilutions of the starting standard of cannabidiol (CBD_{std}) methanolic solution (1 mg mL^{-1}), or delta-9-tetrahydrocannabinol (Δ^9-THC-D_3) methanolic solution (0.1 mg mL^{-1}), were prepared in the range 0.001–0.1 mg ml^{-1}. Standard solutions were injected three times in UPLC system and monitored at 225 nm. Both compounds were eluted as single sharp peaks at retention time of 2.9 min for CBD_{std} and 5.8 min for Δ^9-THC- D_3. In the operative concentration range the trend was linear, with no saturation effects that could bend the linearity.

The area under each peak was quantified by instrumental software and plotted *versus* the concentration. The best fit of experimental data in the plot "Peak area *vs* [CBD] or [Δ^9-THC]" displayed in Fig. 1 was, as expected, a straight-line, represented by the following mathematical equations: $y = (7.96 \cdot 10^7 \pm 3 \cdot 10^5)\,x + (2 \cdot 10^4 \pm 1 \cdot 10^4)$ for CBD standard solutions and $y = (5.473 \cdot 10^7 \pm 5 \cdot 10^4)\,x + (1.9 \cdot 10^4 \pm 2 \cdot 10^3)$ for Δ^9-THC-D_3 standard solutions. Linearity was assessed through evaluation of the coefficient of determination, which should be greater than 0.998; in both cases it was equal to 0.999.

Limit of detection (LOD) and limit of quantification (LOQ) were estimated *via* calibration approach following the JRC (Joint Research Centre) guidelines (Schaechtele and Robouch, 2016). Limit of detection and limit of quantification for CBD were 1 μg mL^{-1} and 4 μg ml^{-1}, respectively. Limit of detection and limit of quantification for Δ^9-THC- D_3 were 0.4 μg ml^{-1} and 1 μg ml^{-1}, respectively. On the basis of these results, the calculated equation of the regression line was then employed to determine the CBD and Δ^9-THC concentration in the extracts.

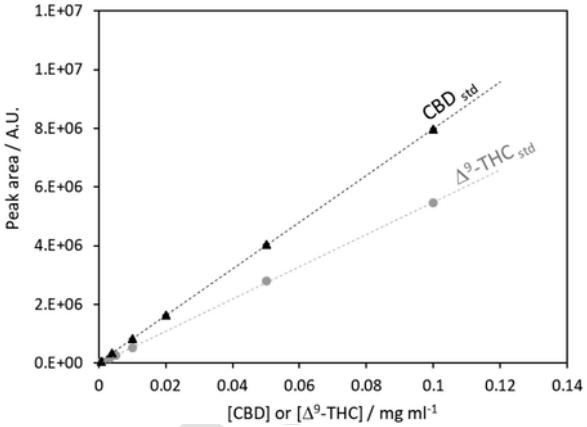

Fig. 1. Plots of cannabidiol (CBD) or delta-9-tetrahydrocannabinol (Δ^9-THC) standard solutions concentrations *versus* the area under the respective assigned peak, as detected by ultra performance liquid chromatograms at 225 nm. Dotted lines are the regression lines.

3.2. Conventional solvent extraction versus supercritical extraction

Many conventional and non-conventional solvent-based procedures have been already performed in the literature targeting cannabinoids from hemp. In this work the general, fast and common alcoholic solvent maceration method was selected in order to set a benchmark and compare results from supercritical methodologies (Fiorini et al., 2019; Monton et al., 2019; Mudge et al., 2017). By conventional methanolic extraction, the collected product was a green oil (shown in Fig. 2) recovered with 22 % yield. The black solid line in Fig. 2 displays the UPLC-UV-MS chromatogram recorded at 225 nm. Focusing on the first 5 min, the chromatographic profile displays a main peak at 3.3 min (peak 2), following a less intense peak at 2.9 min (peak 1). By means of mass spectrometry it was possible to assign peak 1 to cannabidiol (CBD, m/z = 315.2, MW = 314.46 g mol^{-1}) and peak 2 to cannabidiolic acid (CBDA, m/z = 359.1, MW = 358.47 g mol^{-1}).

When performing supercritical extraction, a brown-yellow oily extract was collected (picture displayed in Fig. 2). The black dotted line in Fig. 2 displays its relative UPLC-UV-MS chromatogram. The chromatographic profile at 225 nm is identical to what detected for the conventional methanolic extract, except for the higher relative intensity of the CBD peak (peak 1).

The sc–CO_2 extraction curve "yield *vs* consumed CO_2" (function of extraction time) is displayed in Fig. 3. The extraction yield reaches a plateau in correspondence of the value 14 %. This number, pointing out that the yield of sc–CO_2 is lower than the conventional methanolic extract yield, is in agreement with literature data dealing with supercritical extractions (da Silva et al., 2016). In fact, sc–CO_2 is generally a more selective technique than conventional solvents methods, sometimes characterized by lower yield, but able to provide higher purity extracts.

As a first consideration, the methanolic extract, green in color, together with the expected cannabinoids, contains chlorophylls. Most importantly, the CBD content was quantified and results are displayed in Table 1. The CBD content in the sc–CO_2 extract was higher, confirming the enhanced selectivity of the technique. As a main difference in the chromatographic profiles recorded at 225 nm, the ratio peak 1/peak 2 in the sc–CO_2 chromatogram increased, pointing out a higher content of CBD with respect to CBDA. This behavior is explained by the decarboxylation kinetics that is favored by a temperature increase. During the supercritical extraction the temperature was set at 60 °C and main-

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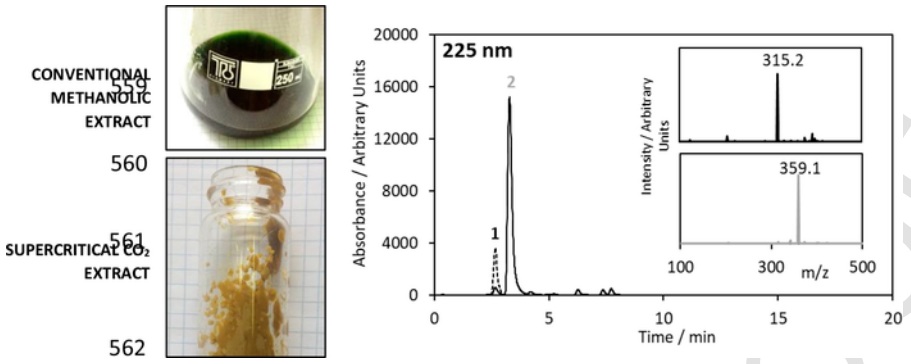

Fig. 2. Left: pictures of extracts obtained through conventional methanolic extraction and supercritical CO_2. Right: Ultra performance liquid chromatograms recorded at 225 nm when injecting the conventional extract (black solid line) and the supercritical CO_2 extract (black dotted line). Results from mass spectroscopy of peak 1 (black) and peak 2 (grey) are shown in the insets.

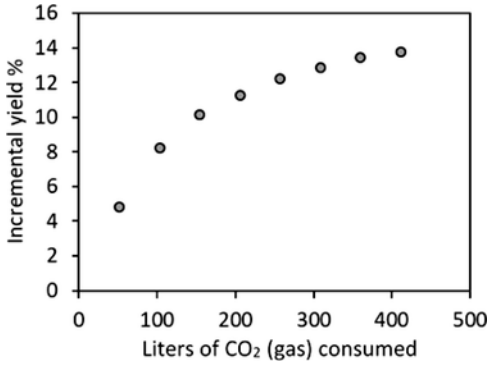

Fig. 3. Supercritical CO_2 extraction profile: each point represents the incremental yield (% mass $_{extract}$/mass $_{biomass}$), detected over time and hence over CO_2 consumption.

tained throughout the experiment. Cannabidiolic acid decarboxylation phenomenon, yielding CBD as product, takes place in the sc–CO_2 extraction vessel in a much higher extent compared with conventional methanolic extraction, where the temperature was the ambient one.

3.3. Heat treatment studies

It has been already clarified by the literature that CBD is not a biosynthetic product of *Cannabis sativa* L. plant. In fact, the plant synthesizes cannabigerolic acid (CBGA) from olivetolic acid and geranyl pyrophosphate. Then, enzymes such as cannabidiolic acid-synthase (CBDA-synthase) catalyze CBGA transformations leading to cannabidiolic acid (CBDA). CBDA is therefore the main product of "legal" fiber hemp plant (Citti et al., 2018; Sirikantaramas and Taura, 2017). This explains the presence of the main peak of CBDA in the extracts (see Fig. 2A).

Aiming at targeting CBD as the main product, in agreement with recent publications studying the decarboxylation kinetics of CBDA yielding CBD, some heat treatments experiments were performed on the pulverized starting material. It was purposely avoided to perform heat-treatments later on the extracts, in order to prevent any decomposition/degradation effect which is more likely to occur on "concentrated products" than in the presence of the matrix.

Heat treatments were performed on the starting pulverized biomass at 100 °C, in order to maximize CBDA conversion into CBD, without any adverse effect on the products themselves. Following the conclusion of decarboxylation studies by Citti et al., 100 °C was identified as the optimum temperature guaranteeing the formation of CBD as the only product of decarboxylation. At 100 °C or above, other processes could be involved, including the formation of unknown side products suggesting a more complex chemistry behind (Citti et al., 2018). On the other hand, about the duration of the heat-treatment, there is no general consensus, being the decarboxylation efficiency dependent on the particle size, eventual air flow, *etc*. For this reason, some trials were conducted changing the heat treatment duration from 1 to 2 and 6 h.

The heat-treated biomass then underwent conventional methanolic extraction in order to analyze the product and study the effect of the temperature on the conversion of CBDA into CBD. Results are displayed in Fig. 4. Notably, the increase of the duration of the heat-treatment was able to decrease the intensity of the CBDA peak and, in parallel, increase the intensity of CBD peak. 6 h treatment at 100 °C were found necessary to convert almost all CBDA into CBD by decarboxylation. A residue of the acidic form is still detectable, but the amount is negligible and it was not worth to further prolong the heat treatment.

These data confirm that the higher content of CBD detected in the supercritical extract on the pristine biomass (Fig. 2 and entry 2 in Table 1) was due to a partial decarboxylation reaction occurred during the extraction period when the vessel was maintained at 60 °C.

Once the best conditions for the heat-treatment were optimized, the supercritical extraction was repeated on the biomass, pre-treated for 6 h at 100 °C. From UPLC results, the CBD content was quantified and it was found equal to 50.2 % of the extract, corresponding to 6.21 % by weight of the dry biomass (entry 4 in Table 1). It should be mentioned that the CBDA (and hence CBD) content is strongly dependent on the variety of the hemp plant (Perrotin-Brunel et al., 2010). *Cannabis sativa* L. varieties grown for fiber uses are generally poor sources of cannabinoids, including CBD (lower than 4%) (Mead, 2017). Molecular studies of chemotype have introduced innovative varieties

Table 1
Summary of the main results from conventional methanolic and supercritical CO_2 extractions.

Sample	Extraction procedure	Heat treatment	Extraction Yield %	Extract appearance	CBD * % w/w $_{on\ dry\ biomass}$	CBD % w/w $_{on\ extract}$	% CBD/CBDA*
1	Conventional methanolic extract	–	22	Green oil	0.71 ± 0.01	3.0 ± 0.5	6
2	sc-CO_2*	–	14	Yellow-brown oil	2.22 ± 0.04	15.8 ± 0.3	23
3	Conventional methanolic extract	100 °C - 6 h	17	Green oil	5.43 ± 0.03	45.2 ± 0.2	98
4	sc-CO_2	100 °C - 6 h	13	Yellow-brown oil	6.21 ± 0.02	50.2 ± 0.2	98

* CBD: cannabidiol, CBDA: cannabidiolic acid, sc–CO_2: supercritical CO_2.

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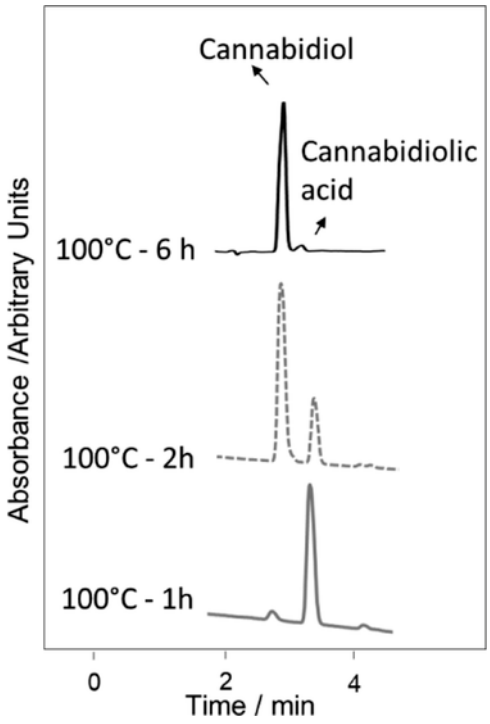

Fig. 4. Ultra performance liquid chromatograms recorded at 225 nm: comparison of samples extracted after different heat-treatments durations of the biomass at 100 °C.

with preferential methabolic pathways, able to increase the synthesis of certain cannabinoids respect to others, depending on the application (Pertwee, 2014). In terms of order of magnitude, the amount of CBD detected in this work, deriving from CBDA decarboxylation, is in agreement with literature data and with data provided by the grower, suggesting an almost complete recover of the target compound from the starting plant material (Grijó et al., 2018; Pacifici et al., 2017).

Decarboxylation phenomena should be taken into account when selecting the technique for cannabinoids analysis. In the literature some works make use of gas chromatography with a FID detector to analyze extracts from hemp flowers. Due to the high temperatures reached during the detection process, the decarboxylation takes place directly *in situ*, giving rise to blunders in the qualitative determination, and hence in the following quantification. CBD is detected in place of CBDA, Δ^9-THC is detected in place of Δ^9-TH*CA.ca.* Gas chromatography with FID detection is the preferable technique in the volatile fraction analysis (essential oils, terpenes, fatty acids after derivatization) while, for the non-volatile fraction, liquid chromatography should still be preferred (Chawla and Kunnen, 2005; Da Porto et al., 2015; Grijó et al., 2019a). In some cases, the goal is to measure simultaneously the acidic and the decarboxylated forms; in these circumstances GC-FID is the correct technique to be applied and the result is given as the total amount of CBD and CBDA forms (or Δ^9-THC and Δ^9-THCA forms) (Chawla and Kunnen, 2005).

3.4. Cannabidiol extraction and purification process

Considering the previous results, an optimization of the extraction and purification process is proposed. An overview of the process flowchart is displayed in Fig. 5. Detailed results on CBD content are shown in Table 2. The suggested extraction/purification downstream processes include a first step aiming at getting a higher surface-to-volume ratio of the starting biomass, easing the decarboxylation kinetics and the following extraction processes. Then heat treatment enables to fasten the kinetics of decarboxylation of CBDA to CBD, without any adverse effect on the other side reactions occurring. By step 3, a supercritical extract was obtained, characterized by a CBD content of about 50 % (Table 2, entry 4). Subsequently, a winterization procedure was then carried out, proved to be efficient in removing the waxes from crude extracts: 6% of waxes were successfully separated from the oily extract. This is in agreement with data from the literature indicating a waxes content from 5 to 10 % (Attard et al., 2018). By the winterization process, an increase of the CBD content up to 53 % was achieved (Table 2, entry 5). Flash chromatography was finally successfully employed for a further purification, able to separate the undesired compounds from the target one. A final product, characterized by a CBD content of 79 % (Table 2, entry 6a) was obtained. The chromatogram

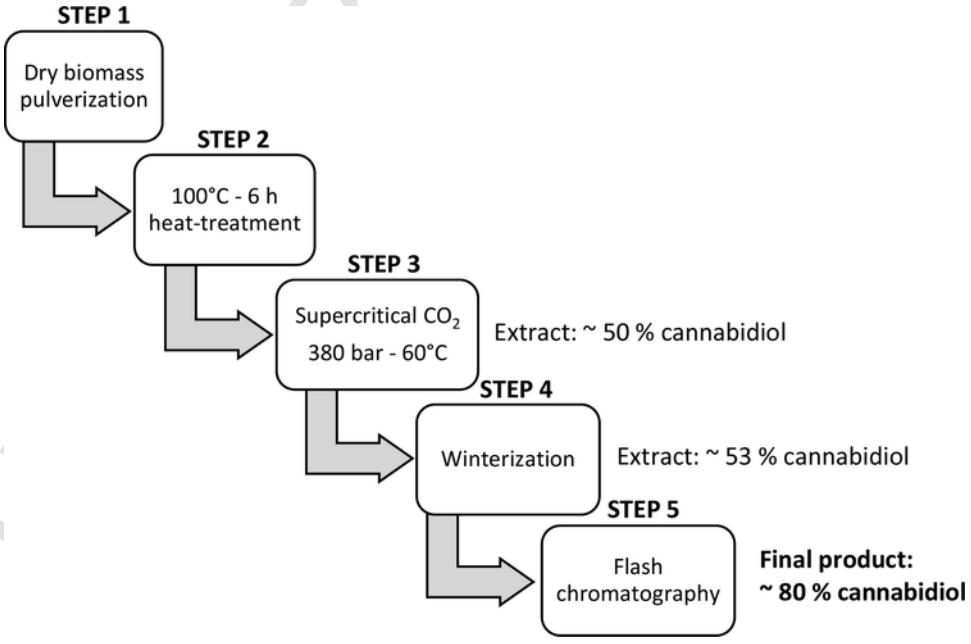

Fig. 5. Process flowchart: cannabidiol extraction and purification downstream process from the native inflorescences to the final cannabidiol-enriched and delta-9-tetrahydrocannabinol-free extract.

Table 2
Cannabidiol and delta-9-tetrahydrocannabinol content in the samples at different purification stage.

Sample	Extraction/purification stage	CBD * % w/w $_{on\ extract}$	Δ^9-THC * % w/w $_{on\ dry\ biomass}$	Δ^9-THC % w/w $_{on\ extract}$
4	sc-CO_2 * extract after 100 °C - 6 h heat treatment	50.2 ± 0.2	0.370 ± 0.001	3.01 ± 0.01
5	Winterized extract	53.2 ± 0.3	0.375 ± 0.002	2.98 ± 0.03
6a	After flash chromatography, adsorption on Celite	79.0 ± 0.4	n.d.	n.d.
6b	After flash chromatography, adsorption on C18	78.4 ± 0.3	n.d.	n.d.

* CBD: cannabidiol, Δ^9-THC: delta-9-tetrahydrocannabinol, sc–CO_2: supercritical CO_2.

of the final product, recorded at 225 nm, is displayed in Fig. 6. Among the two different materials used for pre-loading the sample, Celite was found to be more recommendable than C18 silica. This last material in fact enabled the separation CBD from other species (the final product was characterized by a CBD content of 78 %) but, being more expensive than Celite, in the view of scale up, it was not further considered.

Many works in the literature have been already published on the extraction of cannabinoids from hemp and on the related analytical tools and methodologies able to qualitatively and quantitatively determining the species in the extract (Brighenti et al., 2017; Citti et al., 2018; Da Porto et al., 2014; Montserrat-De La Paz et al., 2014; Pacifici et al., 2017). Many of them, already employed supercritical CO_2 for a faster and more selective extraction (Aladić et al., 2015; Attard et al., 2018; Grijó et al., 2019b, 2018; Rovetto and Aieta, 2017). The academic research interest around hemp-derived oil is however decreasing also due to the number of practical applications, patents and already marketed products.

On the other hand, the specific focus on cannabidiol is increasing in recent times (Hacke et al., 2019). Its non-psychotropic nature, together with its high content obtainable from the plant and its beneficial effects towards many diseases (epilepsy and pain-relief mainly), has been putting the attention on this compound (Costa et al., 2007; Herlopian et al., 2020; Klier et al., 2020). Recently the FDA approved the first ever cannabis-derived medicine for these conditions, Epidiolex, which contains CBD. As an example from the literature, hydrodistillation and steam distillation methods have been assessed by Fiorini et al. working on hemp byproducts aiming at essential oils production containing CBD, together with other bioactives (Fiorini et al., 2019). Other papers in the literature have been focused on CBD extraction by supercritical fluids. Moreno et al. and Aiello et al. recently published a strategy to recover cannabinoids from hemp by using pressurized and supercritical CO_2. However, in many cases yields were lower and no further purification strategies have been developed (Aiello et al., 2019; Moreno et al., 2020).

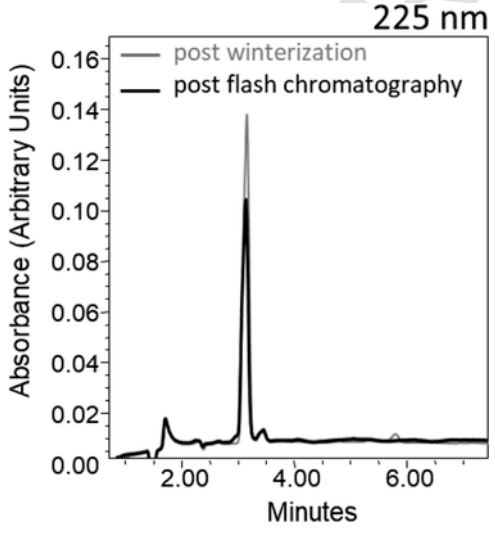

Fig. 6. Ultra performance liquid chromatograms recorded at 225 nm injecting samples before (grey line) and after (black line) separation by flash chromatography.

In this work, a comprehensive scalable strategy, able to assess the experimental conditions for each step, was successfully carried out targeting cannabidiol as the specific compound. A final product, containing almost 80 % of CBD, was successfully obtained. This product, even at this grade of purity, could be industrially appealing both in terms of composition, which can anyway induce the therapeutic action, and as production process since it has been achieved by a combination of green and scalable technologies.

At present, many products are in fact commercialized with a certain and specific % CBD depending on the target application (pharmaceutical/nutraceutical, *etc.*). Some preparations span from "low" levels of CBD (around 8–10 %) up to pure crystals. Considering all these aspects, the final proposed product can represent either the final target, or the starting point for more specific procedures, able to yield the pure crystals, if needed for more specific applications.

3.5. Delta-9-tetrahydrocannabinol quantification

The psychoactive potency of hemp is expressed in terms of the total Δ^9-THC content of the dried upper parts of the plant. The sum of Δ^9-THC and THCA content is limited to 0.2 % w/w by EU regulations (Council Regulation (EC) No. 1420/98) and settled by law in many countries also outside the European Union (Mechtler et al., 2004). In Italy the most recent law for industrial hemp (242/2016) states that only for farmers this limit is extended to 0.6 % (Cas et al., 2019). The legislation framework is confusing concerning whether the derived products can be sold or not, so it is still very important to assess the legality of hemp, determining the total amount of THC in Cannabis derivatives. In addition, it should be mentioned that when handling "legal" hemp species to get derivatives such as plant extracts, the illegal portion results concentrated in the extract or in the starting biomass, increasing its presence at least in one fraction.

Aiming at studying the entire extraction process in terms of psychoactive fraction content, the analysis of all the derivatives of all steps, including the wastes, was carried out.

First, Δ^9-THC was identified in the chromatograms of the extracts displayed in Fig.2. The molecular weight of Δ^9-THC is the same of CBD one. For this reason, together with mass spectrometry, a Δ^9-THC standard was co-injected in order to confirm that the corresponding peak was the one eluting at 5.8 min. By means of the calibration line, build with standard solutions, the Δ^9-THC content in the extracts was quantified. Results are displayed in Table 2.

Extracts deriving from pristine biomass, without heat-treatment, were not analysed in terms of Δ^9-THC content because, under the light of decarboxylation results, the presence of Δ^9-THCA, the acidic form of Δ^9-THC, could affect the final data.

The analysis of sample 4, the oil extracted in supercritical conditions, starting from the heat-treated biomass, revealed that the extract was enriched by almost 3% in Δ^9-THC, corresponding to 0.37 % by weight of the starting biomass. This is in agreement with data provided by the farmer. A further conventional methanolic extraction was conducted on the residual biomass, but no Δ^9-THC was detected. This means that the supercritical conditions procedure effectively extracted almost all the psychoactive fraction, together with the target com-

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pound, CBD. This is obviously a drawback, needed to be solved in the purification procedure.

Sample 5, deriving from the winterization procedure, displayed the same Δ^9-THC content of the non-winterized sample 4, as expected. The winterization procedure is only effective in waxes removal, leaving unchanged the alcoholic-soluble fraction, containing CBD and Δ^9-THC.

Flash chromatography has been already used in the literature specifically for the separation of the psychoactive fraction from the other cannabinoids (Wohlfarth et al., 2011). Interestingly, both samples deriving from purification through flash chromatography, either pre-adsorbed on Celite or on C18 silica powder, were successfully deprived of Δ^9-THC, which was left behind in the other discarded fractions without any need for further treatments. The results can be easily visualized in Fig. 6, showing the UV-chromatograms before and after flash chromatography. The final sample did not show any more residues of the Δ^9-THC psychoactive compound, eluting at 5.8 min, when present in the extract. Flash chromatography was therefore also successful in separating the undesired psychoactive fraction from the targeted CBD-enriched oil.

4. Conclusions

To the best of authors' knowledge, up to now no comprehensive studies have been published proposing a downstream process, making use of green and scalable technologies, such as supercritical CO_2 extraction, able to provide a medium-high purity CBD-enriched product.

Overall, from the results presented in this article it can be concluded that:

- Heat treatment of the pristine biomass enables to fasten the kinetics of decarboxylation of CBDA to CBD, without any adverse effect on the other side reactions occurring.
- The feasibility of a "green" and scalable strategy such as supercritical CO_2 extraction was assessed, enabling a selective extraction of Cannabis oil, without any use of toxic and unsafe organic solvent.
- Further purification steps, performed first by winterization and then by flash chromatography, were able to enhance the CBD purity of the extract up to almost 80 % by weight.
- The psychotropic fraction, getting concentrated in the supercritical CO_2 extract and represented by the presence of Δ^9-THC, was successfully eliminated during the flash chromatography purification stage.

Even though some applications might need higher purity products, the present work could represent an advance with respect to purely analytical or lab-scale researches, aiming at providing a scalable protocol.

In the view of assessing software-designed approaches and reach higher levels of optimization, mathematical modelling supported by design of experiment could represent the step further to get a complete plan when scaling up the overall process to industrial plants.

CRediT authorship contribution statement

Stefania Marzorati: Conceptualization, Methodology, Data curation, Validation, Writing - review & editing. **Luisella Verotta:** Project administration, Conceptualization, Supervision.

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.

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