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Innovative methods for the preparation of medical *Cannabis* oils with a high content of both cannabinoids and terpenes

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ABSTRACT

Cannabis-based medications are being increasingly used for the treatment of different clinical conditions. Among all galenic formulations, olive oil extracts from medical *Cannabis* are the most prescribed ones for their easy preparation and usage. A great variety of methods have been described so far for the extraction of medical *Cannabis* oils to reach a high yield of Δ^9-tetrahydrocannabinol (Δ^9-THC), but poor attention has been paid to the preservation of the terpene fraction from the plant, which may contribute to the overall bioactivity of the extracts.

In this context, the present study was aimed at the chemical characterization of different medical *Cannabis* oils prepared by following both innovative and existing extraction protocols, with particular attention to cannabinoids and terpenes, in order to set up a suitable method to obtain an extract rich in these chemical classes. In particular, six different extraction procedures were followed, based on different techniques, of which all but one included a decarboxylation of the plant material. The profile of cannabinoids was studied in detail by means of HPLC-ESI-MS/MS, while terpenes were characterized by means both GC-MS and GC-FID techniques coupled with solid-phase microextraction operated in the head-space mode (HS-SPME).

An innovative method that is based on the extraction of the oil by dynamic maceration at room temperature from plant inflorescences, which were partially decarboxylated in a closed system at a moderate temperature and partially pre-extracted with ethanol, produced similar yields of bioactive compounds as that obtained by using a microwave-assisted distillation of the essential oil from the plant material, in combination with a maceration extraction of the oil from the residue. Both these new methods provided a higher efficiency over already existing extraction procedures of medical *Cannabis* oils and they can be applied to obtain a product with a high therapeutic value.

1. Introduction

Cannabis sativa L. is an annual cycle herbaceous plant belonging to the *Cannabinaceae* family and to the order of Urticales [1]. The main classes of compounds present in this plant are cannabinoids, terpenes and flavonoids. Cannabinoids are a class of terpenophenolics, derived from the alkylation of an alkyl resorcinol with a monoterpene unit [2]. They are mainly synthesized in glandular trichomes, which are more abundant in female inflorescences [3]. Among them, the most representative compounds are Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA), cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA) (Fig. 1). These native acidic cannabinoids undergo a spontaneous decarboxylation under the action of light and heat, leading to formation of their neutral counterparts, including Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD) and cannabigerol (CBG) [4].

Many studies have demonstrated the role of Δ^9-THC in the analgesia for spasticity associated with pain diseases and in chronic

Abbreviations: Δ^9-THCA, Δ^9-tetrahydrocannabinolic acid; CBDA, cannabidiolic acid; CBGA, cannabigerolic acid; Δ^9-THC, Δ^9-tetrahydrocannabinol; CBD, cannabidiol; CBG, cannabigerol; Δ^9-THC-d_3, deuterated Δ^9-tetrahydrocannabinol; 11-OH-Δ^9-THC-d_3, deuerated 11-hydroxy-Δ^9-tetrahydrocannabinol; Δ^9-THC-COOH-d_3, deuterated Δ^9-tetrahydroxycannabinolic acid; HCOOH, formic acid; ACN, acetonitrile; MeOH, methanol; EtOH, ethanol; H_2O, water; CAD, collision-activated dissociation; CBN, cannabinol; SD, standard deviation.

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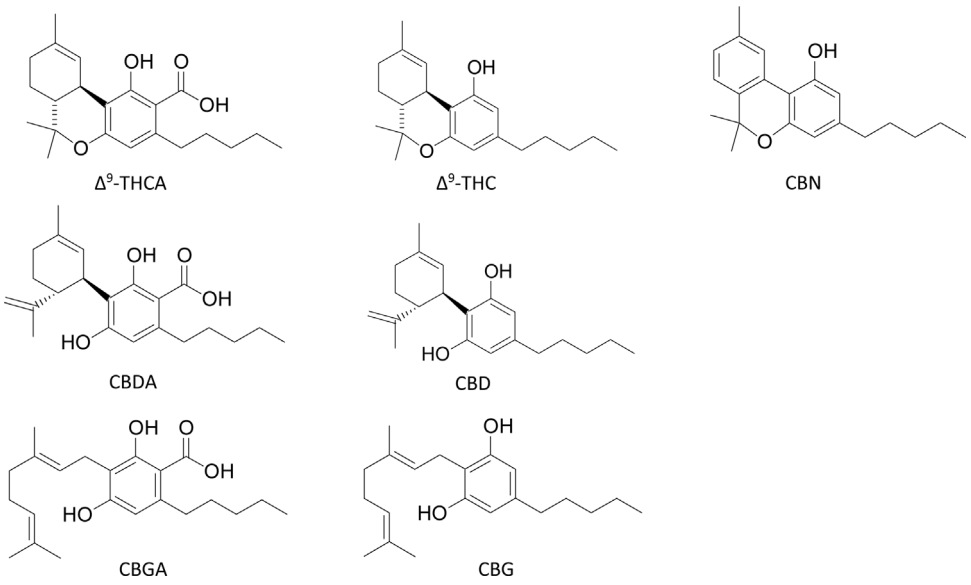

Fig. 1. Chemical structures of the main cannabinoids present in the inflorescences of medical *Cannabis*.

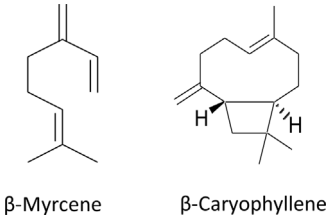

Fig. 2. Chemical structure of the main monoterpene and sesquiterpene present in the inflorescences of medicinal *Cannabis*.

pain resistant to non-steroidal anti-inflammatory drugs, corticosteroids or opioids; it is also a valuable therapeutic agent against chemotherapy-related nausea and vomiting, cachexia and anorexia in patients with cancer or AIDS, glaucoma resistant to conventional therapies and also in the reduction of facial and body movements in Gilles de la Tourette syndrome [5]. Δ^9-THC is the main compound present in *Cannabis* varieties used for either medical or recreational purposes [6]. Besides Δ^9-THC, CBD displays several biological activities, such as antioxidant, anti-inflammatory, anti-microbial [7–9] and neuroprotective activity [10], related to the action on different targets [7]. CBD is mainly present in fiber-type *C. sativa* varieties, also known as industrial hemp or hemp, which is traditionally used in the food and textile ambits [6,11,12]. Hemp has a Δ^9-THC content lower than the legal limit of 0.2−0.3% [11–13]. CBD has also been recently detected in honey produced from hemp [14].

Cannabinoids do not represent the only active compounds in *Cannabis* inflorescences. Indeed, terpenes are another well represented class of compounds biosynthesized in the plant [8,11]. Among terpenes, β-myrcene, limonene, *trans*-ocimene and α-terpinolene are the most abundant monoterpenes in *Cannabis* inflorescences, while β-caryophyllene and α-humulene are the most represented sesquiterpenes [8,11] (Fig. 2).

A hypothesis of a synergistic effect between cannabinoids and terpenes has been postulated, in view of the so-called "*entourage effect*" [15]. Up to date, no reliable scientific evidence of this synergy exists, at least at the cannabinoid (CB) receptor level [15,16]. Nonetheless, it would be premature to deny the existence of either pharmacodynamic or pharmacokinetic interactions among active compounds present in *Cannabis*, as many biological activities have been ascribed to its terpenes, including anti-inflammatory, analgesic and anxiolytic properties [17]. Therefore, it is important to look for extraction methods which allow the preservation of *Cannabis* terpenes in the products used for therapeutic purposes.

Medical *Cannabis* preparations can be used either orally or by inhanation [18]. As regards medical *Cannabis* oils for therapeutic use, various extraction methods have been described in the literature [19–21]. It should be pointed out that, in relation to the preparation of medical *Cannabis* oils, no attention is usually paid to the volatile components of the plant. Indeed, the decarboxylation process, that is frequently applied to the plant material to convert cannabinoic acids into neutral cannabinoids, can cause the complete loss of this fraction. Since oil preparation conditions can affect the final product composition, the development and optimization of an efficient procedure to be followed by pharmacists becomes crucial in order to obtain a final product of high quality and to ensure the reproducibility of its therapeutic effects.

In the light of all the above, the present study was aimed at the chemical characterization of different medical *Cannabis* oils obtained with both innovative and existing extraction protocols in order to develop a highly efficient method for both cannabinoids and terpenes. Six different extraction methods were followed, based on different techniques, of which all but one include a decarboxylation of the plant material. The analysis of cannabinoids in the oils was evaluated by means of HPLC-ESI-MS/MS, while terpenes were analysed by means both HS-SPME-GC-MS and GC-FID techniques coupled with solid-phase microextraction in the head-space mode (HS-SPME).

2. Material and methods

2.1. Chemicals and solvents

Standard solutions of cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), cannabidiol (CBD) and cannabigerol (CBG) were purchased from Cerilliant (Round Rock, TX, USA). Standard solutions of Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA), Δ^9-tetrahydrocannabinol (Δ^9-THC), the internal standards, including deuterated Δ^9-tetrahydrocannabinol (Δ^9-THC-d_3), deuerated 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-Δ^9-THC-d_3) and deuterated Δ^9-tetrahydroxycannabinolic acid (Δ^9-THC-COOH-d_3), and ammonium acetate were provided by Sigma-Aldrich (Milan, Italy). Formic acid (HCOOH), acetonitrile (ACN), methanol (MeOH) and ethanol (EtOH) HPLC grade were purchased from

Table 1
MRM transitions of target cannabinoids.

Peak number	Compound	Retention time (t_R, min)	MRM transitions	Collision energy (eV)	Ion spray voltage (V)	Dwell time (msec)
1	CBDA	5.4	357 → **245**, 179[b], 271[b]	37, 34, 36	−4500	100
2	CBGA	5.8	359 → **341**, 315[b], 297[b]	29, 33, 38	−4500	100
3	Δ^9-THCA	6.4	357 → **191**, 245[b]	45, 41	−4500	100
4	CBG	9.9	315 → **136**, 191[b], 177[b]	36, 33, 33	−4500	100
5	CBD	9.9	313 → **245**, 107[b]	30, 48	−4500	100
6	Δ^9-THC	11.0	313 → **245**, 191[b], 203[b]	37, 38, 49	−4500	100
IS1	Δ^9-THC-d_3	10.5	316 → **194**, 248[b], 182[b]	37, 38, 35	−4500	100
IS2	11-OH-Δ^9-THC-d_3	7.7	332 → **270**, 314[b]	45, 25	−4500	100
IS3	Δ^9-THC-COOH-d_3	5.5	346 → **248**, 302[b]	38, 28	−4500	100

Experimental conditions as in Section 2.4.
[a] Quantifier transitions are highlighted in bold.
[b] Qualifier transitions used for confirmation.

PanReac Applichem (Barcellona, Spain). Water (H_2O) was purified using a Milli-Q Plus185 system from Millipore (Milford, MA, USA). Extra virgin olive oil was of European Pharmacopoeia grade.

2.2. Plant material and extraction of Cannabis oils

C. sativa inflorescences belonging to the Bedrocan® variety were available at the Dr. Marco Ternelli's Pharmacy in Bibbiano, Reggio Emilia, Italy. This medical *Cannabis* plant material of Dutch origin is certified for a content of Δ^9-THC of 22 % (w/w), while the amount of CBD is lower than 1%. The corresponding oils were prepared in the Pharmacy by Dr. Ternelli on medical prescription requests, while the analyses were carried out at the Toxicology Laboratory of the Forensic Institute of the Department of Biomedical, Metabolic and Neurosciences of the University of Modena and Reggio Emilia, according to Italian DM 9/11/2015 to obtain the cannabinoid concentrations.

Cannabis inflorescences were crushed before the decarboxylation step and the extraction with olive oil. All medical *Cannabis* oils were prepared using a drug-to-solvent ratio of 1:10 (w/v). In particular, oil O1 was extracted according the protocol described by Romano and Hazekamp [21]. Briefly, the inflorescences were mixed with olive oil and the mixture was heated in a water bath at 98 °C for 120 min. Oil O2 was prepared according to the procedure developed by the Italian Society of Preparatory Pharmacists (SIFAP), which is not published in the scientific literature. In particular, the plant material underwent a decarboxylation step in an oven at 115 °C for 40 min; subsequently, olive oil was added and a turbo-extraction was performed for 3 min, followed by a maceration of the mixture in water bath at 98 °C for 40 min. Oils O3 and O4 were prepared following the extraction procedures described by Pacifici et al. and Calvi et al. [19,20], respectively. In both these methods, plant inflorescences were initially heated at 145 °C for 30 min for the decarboxylation of native cannabinoic acids. In the method described by Pacifici et al. the decarboxylation was followed by a maceration of the plant material in olive oil at 98 °C for 60 min [20]. In addition, Calvi et al. performed the extraction of *Cannabis* inflorescences in olive oil by taking advantage of ultrasound-assisted extraction (UAE) at 35 Hz for 30 min [19]. Oil sample O5 was prepared as follow: firstly, 80 % of the inflorescences weight was submitted to a heating step in an oven in a sealed flask at 115 °C for 90 min. Simultaneously, the remaining 20 % of the inflorescences weight was extracted with EtOH, which was subsequently removed by using a rotary evaporator until a resin/paste was obtained; this residue was then submitted to a heating step in an oven in a sealed flask at 115 °C for 30 min. Then, olive oil was added to the resin/paste and to the inflorescences, and the resulting mixture was left to macerate under mechanical stirring for 24 h at room temperature. Regarding oil O6, a steam distillation at 100 °C for 120 min was performed on 100 g of inflorescences to extract the essential oil. Then, the residual plant material from distillation was pressed to remove residual H_2O and it was extracted with olive oil, as previously described for O4. Finally, the essential oil was added to the olive oil extract. The oil sample O7 was prepared by applying the microwave-assisted technology to the hydro-distillation process. In particular, an ETHOS X apparatus (Milestone Srl, Italy) was used. Eighty g of inflorescences were added to 350 mL of H_2O. For the extraction of the essential oil, the instrument was set as follows: 500 W for 3 min, then 320 W for 10 min and, finally, 290 W for 60 min. The plant material was pressed to remove residual H_2O and the olive oil extract was prepared from the residue as previously described. Finally, the distilled essential oil was added to the oil extract.

For each methods, the extraction was carried out twice.

2.3. Sample preparation for cannabinoid analysis

A portion of 25 mg of *Cannabis* oil were placed into a 10 mL volumetric flask, which was brought to volume with ACN. Then, 100 μL of this solution were diluted 1:50 (v/v) with ACN. Finally, 100 μL of the diluted solution were put into a micro-vial, added with 100 μL of the ISs solution and 100 μL of the mobile phase.

The sample preparation was performed twice for each oil.

2.4. HPLC-ESI-MS/MS analysis of cannabinoids

The HPC-ESI-MS/MS analysis of cannabinoids in the oils was carried out on an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany), equipped with a binary pump, a thermostated autosampler, an online degasser and a thermostated column compartment. The HPLC separation was performed on a Kinetex EVO C_{18} column (100 × 2.1 mm I.D., 5 μm) (Phenomenex, Torrance, CA, USA) [14]. The mobile phase was composed of ammonium acetate (2.0 mM) (A) and ACN (B). The elution conditions were the following: from 30 to 90 % of B in 10 min, then kept at 90 % of B for 5 min, and finally from 90 to 30 % of B in 3 min. The post-running time was set at 2 min. The flow-rate was 0.350 mL/min and the column temperature was 40 °C. The injection volume was 25 μL, using a needle wash between samples. Two injections were performed for each sample.

The MS/MS analysis was performed by using an AB SCIEX API 4000 QTRAP triple quadrupole mass analyzer equipped with an electrospray ionization (ESI) source operating in the negative ion mode. The chromatograms were acquired with the Analyst software (version 1.5.2). The mass analyzer parameters were set as follows: curtain gas 10 psi, collision-activated dissociation (CAD) gas 4 psi, nebulizer gas 35 psi, heater gas 45 psi, nebulizer voltage 4000 V, temperature 450 °C. For the quantitative analysis of cannabinoids, the multiple reaction monitoring (MRM) acquisi-

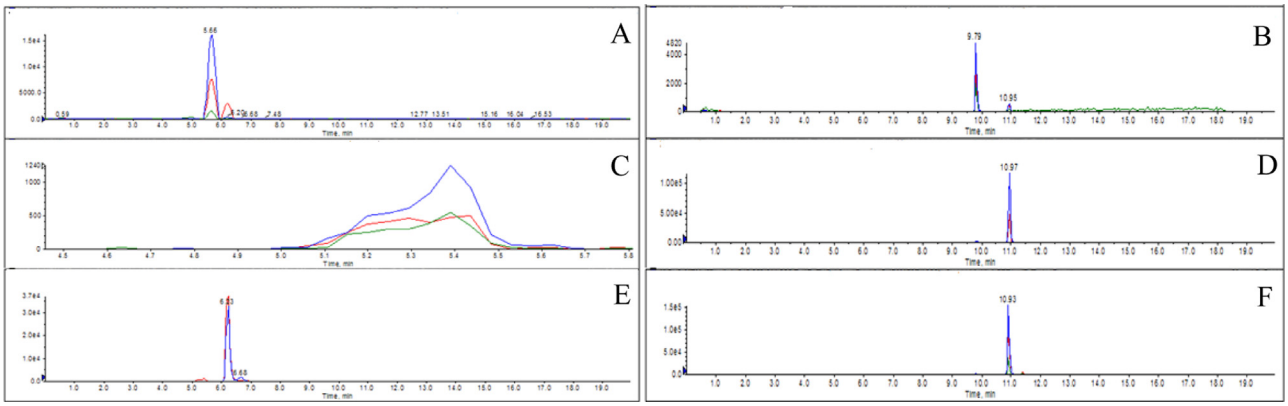

Fig. 3. HPLC-ESI-MS/MS chromatograms of a representative sample of a medical *Cannabis* oil (sample O5). MRM transitions of CBGA (**A**), CBG (**B**), CBDA (**C**), CBD (**D**), Δ^9-THCA (*E*) and Δ^9-THC (**F**).

tion mode was used to ensure high sensitivity and selectivity. The selected transitions are reported in Table 1. A dwell-time of 100 ms was used for all transitions. Among the monitored transitions, for each compound the most intense one was used for quantitative analysis, while the second most abundant one was used as a qualitative transition to confirm the identity of the compound. In addition, the ratio between the areas of the quantitative and qualitative transitions found for reference standards was used to confirm the identity of the analytes in the chromatograms (margin applied: 20 %).

2.5. Sample preparation for terpene analysis

The extraction of terpenes from *Cannabis* oil was carried out by HS-SPME. A manual holder and a 1 cm stable-flex 50/30 μm DVB/CAR/PDMS fibre were employed (Supelco, Bellefonte, PA, USA) [11]. Before GC analysis, the fibre was conditioned in the injector, according to the instructions provided by the manufacturer. A 400 μL amount of medical *Cannabis* oil was placed into a 10 mL flat-bottom vial sealed with a magnetic crimp cap and a PTFE/silicone septum (Supelco). Under the optimized conditions, the sample was heated for 30 min during the equilibrium time in a thermostatic bath at 40 °C. The SPME device was then inserted into the sealed vial by manually penetrating the septum and the fibre was exposed to the headspace for 20 min during the extraction time. After sampling, the SPME fibre was immediately inserted into the GC injector and thermally desorbed. A desorption time of 5 min at 250 °C was used in the split-less mode. Before sampling, the fibre was reconditioned for 5 min in the GC injector port at 250 °C.

The extraction procedure was repeated twice for each sample.

2.6. GC-MS analysis of terpenes

GC-MS analysis of volatile compounds were conducted on a 7890A gas chromatograph, coupled to a 5975C mass spectrometer (Agilent Technologies, Waldbronn, Germany). The column was an Agilent Technologies HP-5-cross-linked poly-5% diphenyl-95 % dimethyl polysiloxane (30 m × 0.32 mm I.D., 0.25 μm film thickness) [11]. The analysis was performed under the following programmed temperature elution: from 45 to 100 °C at 2 °C/min, then raised to 250 °C at 5 °C/min, which was held constant for 5 min. The carrier gas was helium, at a flow rate of 0.7 mL/min. The injector, the transfer line and the ion source were thermo-regulated at 250, 280 and 230 °C respectively. Mass spectra were acquired with an electronic ionization (EI) source at 70 eV, operating in the full-scan acquisition mode in the range of 40-400 *m/z*. MS spectra of eluting peaks were compared with those reported in the NIST mass spectral database (version 1.4)

2.7. GC-FID analysis of terpenes

GC-FID analysis of volatile compounds was performed by using an Agilent Technologies 7820 GC coupled to a flame ionization detector (FID). The column and the oven temperature program were the same as those described in paragraph 2.6. The carrier gas was helium, at a flow rate of 1.0 mL/min. The injector and detector temperatures were set at 250 and 300 °C, respectively.

The relative amount of each volatile compound was expressed as percentage relative peak area on the basis of the total area of the chromatographic peaks.

3. Results and discussion

3.1. Extraction methods for medical Cannabis oils

In this work, different extraction methods were taken into account for the preparation of medical *Cannabis* oil with a high content of both cannabinoids and terpenes.

Firstly, four methods previously described in the literature were applied [19–21]. The first one, described by Romano and Hazekamp, did not include a decarboxylation step of the plant material; indeed, in this case the plant material was extracted by a maceration in olive oil at 98 °C for two hours [21]. In the three subsequent methods, a decarboxylation of the plant material was carried out at 115 °C for 40 min and at 145 °C for 30 min, respectively, followed by an extraction by turbo-emulsion and/or maceration in the first two cases [20] and by UAE in the third one [19].

In addition to these methods, three newly extraction procedures were developed in this work. In the first one, the decarboxylation of the plant material was carried out at a lower temperature (115 °C) for 90 min in a closed flask in order to avoid the loss of volatile compounds. Then, the extraction was performed by dynamic maceration at room temperature. Following this method, a portion of the inflorescences weight was extracted with EtOH to compensate the possible loss of cannabinoids, which are more soluble in EtOH rather than in olive oil. EtOH has indeed been described as the most suitable solvent for the extraction of both cannabinoic acids and their neutral counterparts from the plant material [12]. In the second newly developed method, the essential oil from the plant material was extracted by means of steam distillation, followed by dynamic maceration of the residual plant material in olive oil at room temperature. The essential oil was finally added to the olive oil extract. In the last method, which represents a further improvement with respect with the previous one, the hydro-distillation of the essential oil was performed by using a microwave-assisted apparatus; the conditions applied during this final method were

Table 2
Cannabinoids in medical *Cannabis* oils (O1-O7) analyzed by HPLC-ESI-MS/MS. Data are expressed as mg/mL (mean, $n = 4$) ± SD.

Compound	O1	O2	O3	O4	O5	O6	O7
CBDA	0.8[a]	0.5 ± 0.1	0.3 ± 0.2	0.3[a]	0.6[a]	0.1[a]	0.1
CBGA	0.4 ± 0.1	0.1[a]	–	–	0.1[a]	–	–
Δ^9-THCA	16.7 ± 1.6	0.4 ± 0.1	0.2[a]	–	3.6 ± 1.8	0.8[a]	2.0[a]
CBN	–	0.1[a]	0.2[a]	0.2[a]	0.1[a]	0.3[a]	–
CBG	0.2[a]	0.6 ± 0.1	0.8 ± 0.2	0.7[a]	0.7 ± 0.1	–	–
CBD	0.1[a]	0.1[a]	0.1[a]	0.1[a]	–	0.1[a]	0.1[a]
Δ^9-THC	2.1 ± 0.6	13.2 ± 2.2	11.8 ± 1.8	9.5 ± 2.8	13.0 ± 0.6	15.4 ± 1.7	15.7 ± 1.4

[a] SD < 0.05.

Table 3
Terpenes in medical *Cannabis* oils (O1-O7) analysed by HS-SPME-GC-FID. Data are expressed as % relative peak area (mean, $n = 4$) ± SD.

Peak number	Compound name	*LRI*	O1	O2	O3	O4	O5	O6	O7
1	Butanoic acid	808	0.8 ± 0.1	1.1 ± 0.1	54.8 ± 7.8	–	0.3[a]	0.4 ± 0.1	0.3[a]
2	1-Esanol	856	–	0.6 ± 0.3	–	–	0.5 ± 0.2	0.7 ± 0.2	0.8[a]
3	Methoxy-phenyl-oxime	870	0.6 ± 0.1	3.2 ± 0.2	–	–	3.0 ± 0.2	2.1 ± 0.4	0.8[a]
4	α-Thujene	928	0.5 ± 0.1	0.6 ± 0.1	–	–	0.3 ± 0.2	0.5[a]	0.3[a]
5	α-Pinene	934	1.0 ± 0.3	1.0 ± 0.1	–	–	1.3 ± 0.1	0.9[a]	2.2 ± 0.1
6	β-Pinene	976	1.8 ± 0.9	1.9 ± 0.2	–	–	2.0 ± 0.2	1.6 ± 0.1	1.5[a]
7	2-Octanone	985	3.2 ± 1.2	0.7 ± 0.1	–	–	0.9 ± 0.1	0.5 ± 0.2	0.3[a]
8	β-Myrcene	994	23.7 ± 3.3	26.3 ± 2.1	24.9 ± 6.3	47.6 ± 2.4	28.4 ± 1.4	22.8 ± 0.6	55.8 ± 1.9
9	3-Octanol	998	–	–	–	–	0.2[a]	–	0.2 ± 0.1
10	α-Phellandrene	1005	1.7 ± 0.1	1.9 ± 0.1	–	–	1.7 ± 0.1	1.5[a]	0.9 ± 0.1
11	δ-3-Carene	1010	1.9 ± 0.5	2.0 ± 0.2	–	–	1.9 ± 0.2	1.5 ± 0.2	1.2 ± 0.1
12	α-Terpinene	1017	0.9[a]	1.4 ± 0.2	–	–	1.1 ± 0.3	1.0 ± 0.1	0.8[a]
12	*p*-Cymene	1024	1.5 ± 0.2	1.3 ± 0.1	–	–	1.0 ± 0.1	1.0 ± 0.1	0.9[a]
14	Limonene	1029	6.8 ± 0.8	5.9 ± 0.2	–	11.1 ± 0.4	5.9 ± 0.6	5.9 ± 0.4	4.3[a]
15	1,8-Cineol	1032	–	0.4[a]	–		0.3[a]	0.4[a]	–
16	*cis*-Ocimene	1038	–	0.4[a]	–	–	0.4 ± 0.1	0.3 ± 0.1	0.3[a]
17	*trans*-Ocimene	1049	15.9 ± 1.0	13.8 ± 0.3	–	12.2 ± 1.0	15.7 ± 1.8	14.3 ± 1.6	7.7 ± 0.2
18	γ-Terpinene	1058	1.3 ± 0.1	1.8 ± 0.1	–	–	1.7 ± 0.2	1.6 ± 0.1	1.1 ± 0.1
19	*trans*-Sabinene hydrate	1066	–	–	–	–	0.2[a]	0.3 ± 0.1	0.2[a]
20	α-Terpinolene	1091	31.6 ± 6.3	29.0 ± 2.7	21.9 ± 2.9	24.2 ± 3.0	26.5 ± 1.2	29.2 ± 0.8	11.9 ± 2.4
21	*E*-*p*-2,8-Menthadien-1-ol	1093	–	0.3[a]	–	–	0.3[a]	–	–
22	Linalol	1101	1.3 ± 0.1	1.2[a]	–	–	1.1 ± 0.1	1.6 ± 0.3	1.1[a]
23	Fenchol	1112	–	–	–	–	–	–	0.4 ± 0.1
24	(4*E*,6*E*)-Allocimene	1130	–	–	–	–	0.3[a]	–	0.2[a]
25	1,5,8-*p*-Menthatriene	1135	–	–	–	–	–	0.3 ± 0.1	–
26	Camphor	1146	1.1 ± 0.1	0.5[a]	–	–	0.5[a]	0.7[a]	0.5 ± 0.1
27	Isomenthone	1150	–		–	–	–	–	0.2[a]
28	Menthol	1177	0.5 ± 0.1	0.4[a]	–	–	0.4 ± 0.1	0.5 ± 0.1	0.5[a]
29	Terpinen-4-ol	1186	0.7 ± 0.1	0.5 ± 0.1	–	–	0.4[a]	0.5 ± 0.2	0.3[a]
30	*p*-Cymen-8-ol	1191	0.6 ± 0.1	0.4 ± 0.1	–	–	0.4[a]	0.5 ± 0.1	0.3[a]
31	α-Terpineol	1194	1.0 ± 0.2	0.8 ± 0.1	–	–	0.8 ± 0.1	1.0 ± 0.3	0.6[a]
32	β-Caryophyllene	1424	1.5 ± 0.1	0.8[a]	–	–	0.6[a]	1.1 ± 0.3	1.0[a]
33	α-Bergamotene	1439	–	–	–	–	–	–	0.1[a]
34	Aromadandrene	1443	–	–	–	–	–	–	0.2[a]
35	α-Humulene	1459	0.6[a]	–	–	–	0.2[a]	0.3 ± 0.1	0.3[a]
36	δ-Selinene	1542	–	–	–	–	–	–	0.1[a]
	Total monoterpenes		**93.8 ± 2.1**	**91.7 ± 2.6**	**73.3 ± 1.9**	**95.1 ± 3.3**	**92.7 ± 1.8**	**87.8 ± 1.9**	**92.3 ± 1.6**
	Total sesquiterpenes		**2.1a**	**0.8a**	–	–	**0.8a**	**1.4a**	**0.9a**
	Total area		**98.0 ± 2.2**	**98.1 ± 0.6**	**93.4 ± 11.5**	**95.1 ± 6.9**	**98.1 ± 0.2**	**93.0 ± 4.6**	**97.2 ± 0.3**

[a] SD < 0.05.

carefully optimized by taking into account the effects of microwave power and time.

All the samples were then analyzed for their content of cannabinoids and their profile of terpenes, according to chromatographic methods previously developed and validated in our lab [11,14].

3.2. *Analysis of cannabinoids in medical* Cannabis *oils*

The analysis of cannabinoids in the oils was performed by means of HPLC analysis coupled with tandem mass detection (MS/MS). A representative HPLC-ESI-MS/MS chromatogram of a medical *Cannabis* oil sample is shown in Fig. 3.

The cannabinoids identified and quantified in the samples are shown in Table 2. Cannabinol (CBN) was included among the analytes, being it usually present in degraded or oxidized *Cannabis* products [1,3,13]. In general, all the *Cannabis* oil samples for which a decarboxylation process of the plant material was applied for their preparation had a higher content of Δ^9-THC (9.5–15.7 mg/mL) and a lower amount of its acidic precursor Δ^9-THCA (0.2–3.6 mg/mL). The amount of Δ^9-THCA detected in the samples was dependant on the pre-heating temperature and time. Indeed, *Cannabis* oils O2, O3 and O4 were those with a lower amount of Δ^9-THCA ($\leq$ 0.4 mg/mL), accordingly to the harder decarboxylation conditions applied before the extraction. The only exception to this trend was represented by the oil sample O1, obtained by following the method described by Romano & Hazekamp [21], in which Δ^9-THCA was the predominant compound (16.7 mg/mL); conversely, Δ^9-THC content was 2.1 mg/mL in this sample. This profile resembles that of the fresh plant material, in accordance with the fact that this extraction method did not include a pre-heating step.

All the other cannabinoids analyzed in this study were present in low amount ($\leq$ 0.8 mg/mL), including CBN, whose content was

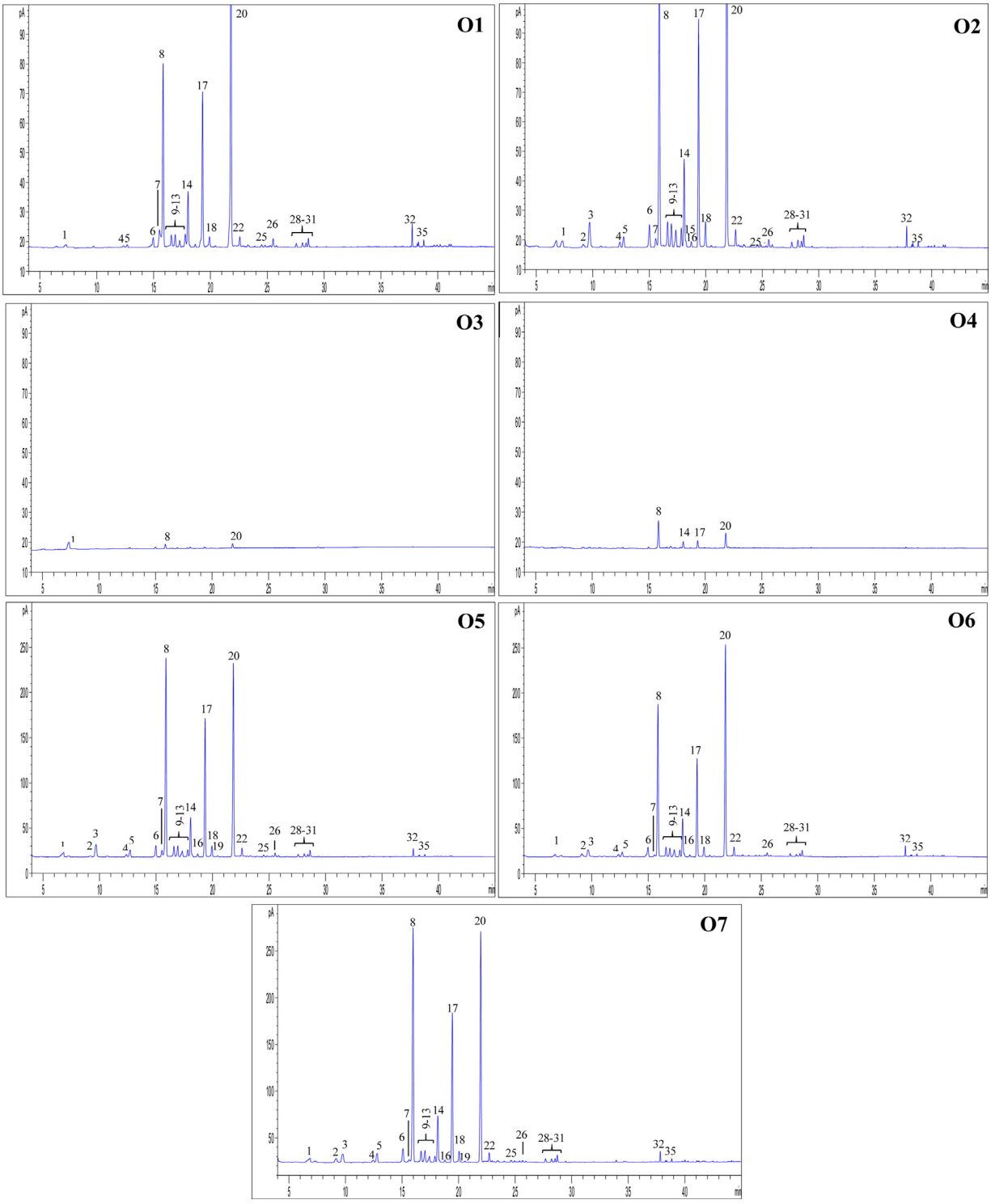

Fig. 4. Representative HS-SMPE-GC-FID chromatograms of medical *Cannabis* oils (samples O1-O7). For peak numbering see Table 2.

$\leq$ 0.3 mg/mL in all the samples, meaning that all the oil preparation procedures employed did not lead to Δ^9-THC degradation or oxidation.

Overall, the best performers in terms of cannabinoids content were found to be *Cannabis* oils O5, O6 and O7, which are those prepared with the extraction methods developed in this work, having a content of Δ^9-THC between 13.0 and 15.7 mg/mL.

The amount of the main cannabinoids in *Cannabis* oil samples from Bedrocan® inflorescences were found to be in agreement with the data available in the literature [19,21,22].

3.3. *Analysis of terpenes in medical* Cannabis *oils*

The analysis of volatile compounds in *Cannabis* oils was performed by means of HS-SPME coupled with both GC-FID and GC-MS. The identification of the compounds was performed by the comparison of the experimental linear retention index (*LRI*) values obtained for the peaks in the GC-FID chromatograms with those reported in the literature for the same plant material [19,23,24]. In addition, GC-MS experiments were carried out in order to confirm the identification of peaks.

The qualitative and semi-quantitative data of the medical *Cannabis* oils is shown in Table 3. In general, the volatiles identified in the oils are in good agreement with those described in the literature for *Cannabis* inflorescences [19,21,23,24]. Monoterpenes were the most represented class of terpenes extracted from the oils, accounting for 73–95 % of total chromatographic peak area. The major peaks belonging to this chemical class were β-myrcene (23.7–55.8 %), α-terpinolene (11.9–31.6 %), *trans*-ocimene (7.7–15.9 %) and limonene (4.3–11.1 %). Sesquiterpenes were detected in low percentage (1-2%), with β-caryophyllene and α-humulene as the main components in this chemical class.

The HS-SPME-GC-FID chromatograms of all the samples analyzed in this work is shown in Fig. 4. In particular, samples O1, O2, O5-O7 displayed a GC profile highly rich in terpenes. Oils O3 and O4 did not follow the same trend of the other samples. As a matter of fact, their volatile profile was found to be extremely poor in its composition: the monoterpenes identified in these samples were β-myrcene, α-terpinolene, *trans*-ocimene and limonene; sesquiterpenes were not detected in these two samples. Curiously, oil O3 was characterised by the presence of a high relative percentage of butanoic acid if compared to all the other samples.

As a general comment, the extraction methods of medical *Cannabis* oils that involve a decarboxylation step at high temperature (145 °C), even for a limited time, led to a poor terpenes fraction. Conversely, the use of milder decarboxylation conditions (115 °C for prolonged time) in a closed flask is highly recommended in order to obtain a product rich in terpenes. A high extraction efficiency for terpenes was also provided by the last two methods, in which the essential oil obtained from the distillation of plant inflorescences was added to the oil extracted from the residual plant.

The chromatographic profile of volatile compounds in these *Cannabis* oils was found to be consistent with previous findings [19,21].

4. Conclusions

In this research work, different extraction procedures were applied to prepare medical *Cannabis* oils and their content of both cannabinoids and terpenes was analyzed in details by HPLC-ESI-MS/MS and HS-SPME-GC-MS/FID, respectively.

This study underlines the importance that extraction parameters cover in the preparation of medical *Cannabis* oil, such as the absence/presence of a decarboxylation step and the temperature/time applied for it. Different extraction conditions led to a different product in terms of both cannabinoids and terpenes. For what concerns cannabinoids, in general, when a decarboxylation step was included in the extraction protocol, the yield in Δ^9-THC raised at the expenses of the native acidic compound Δ^9-THCA. In this view, a pre-heating of the starting plant material is recommended over a direct maceration of inflorescences in olive oil. On the other hand, harsh decarboxylation conditions led to a final product almost deprived of terpenes. The best results in terms of both cannabinoids and terpenes were obtained with a direct maceration extraction of the oil at room temperature from inflorescences previously decarboxylated at a moderate temperature in a closed system and partially submitted to a pre-extraction step with EtOH, which produced similar yields of bioactive compounds as those obtained by using a microwave-assisted distillation of the essential oil from the plant material, in combination with a maceration extraction of the oil from the residue.

Although the "*entourage effect*" of cannabinoids and terpenes has still to be confirmed, the presence of volatile compounds in this galenic preparation may represent an added value as it is well established that they can exert positive effects on human health. Therefore, the extraction protocols developed in this work are highly recommended to obtain medical *Cannabis* oils with a high content of bioactive compounds for therapeutic purposes.

CRediT authorship contribution statement

Marco Ternelli: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing - review & editing. **Virginia Brighenti:** Data curation, Investigation, Methodology, Visualization, Writing - original draft. **Lisa Anceschi:** Investigation, Methodology, Writing - original draft. **Massimiliano Poto:** Investigation, Methodology. **Davide Bertelli:** Software, Visualization. **Manuela Licata:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - review & editing. **Federica Pellati:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing - review & editing.

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