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Comprehensive quality evaluation of medical *Cannabis sativa* L. inflorescence and macerated oils based on HS-SPME coupled to GC–MS and LC-HRMS (q-exactive orbitrap®) approach

Lorenzo Calvi[a,b], Daniela Pentimalli[c], Sara Panseri[a,*], Luca Giupponi[c], Fabrizio Gelmini[d], Giangiacomo Beretta[e], Davide Vitali[a], Massimo Bruno[a], Emanuela Zilio[c], Radmila Pavlovic[a], Annamaria Giorgi[b,c]

[a] Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133 Milan, Italy
[b] Department of Agricultural and Environmental Sciences, Production, Landscape, Agroenergy University of Milan, Via Celoria 2, Milan, Italy
[c] Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas – Ge.S.Di.Mont., University of Milan, via Morino 8, Edolo (BS),Italy
[d] Department of Pharmaceutical Sciences, Università degli Studi di Milano, via Mangiagalli 25, Milan, Italy
[e] Department of Environmental Science and Policy, Università degli Studi di Milano, Milan, Italy

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ABSTRACT

There are at least 554 identified compounds in *C. sativa* L., among them 113 phytocannabinoids and 120 terpenes. Phytocomplex composition differences between the pharmaceutical properties of different medical cannabis chemotype have been attributed to strict interactions, defined as 'entourage effect', between cannabinoids and terpenes as a result of synergic action. The chemical complexity of its bioactive constituents highlight the need for standardised and well-defined analytical approaches able to characterise the plant chemotype, the herbal drug quality as well as to monitor the quality of pharmaceutical cannabis extracts and preparations. Hence, in the first part of this study an analytical procedures involving the combination of headspace-solid-phase microextraction (HS-SPME) coupled to GC–MS and High Resolution Mass-Spectrometry LC-HRMS (Orbitrap®) were set up, validated and applied for the in-depth profiling and fingerprinting of cannabinoids and terpenes in two authorised medical grade varieties of *Cannabis sativa* L. inflorescences (Bedrocan® and Bediol®) and in obtained macerated oils. To better understand the trend of all volatile compounds and cannabinoids during oil storage a new procedure for cannabis macerated oil preparation without any thermal step was tested and compared with the existing conventional methods to assess the potentially detrimental effect of heating on overall product quality.

1. Introduction

Cannabis (*Cannabis sativa L.*) is a highly promising medicinal plant with well documented effectiveness and increasing use in the treatment of various medical conditions [1]. Nonetheless, most of its therapeutic efficacy certainly depends on its phytocannabinoid content and profile comprising over 70 cannabinoids among which (−)-*trans*-Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabigerol (CBG), cannabichromene (CBC), and cannabinol (CBN) are recognised as the most relevant [2]. Above and beyond cannabinoids, a considerable number of substances (around 500) belonging to the terpenes, flavonoids, stilbenoids, fatty acids, alkaloids, carbohydrates, and polyphenols families contribute to the *Cannabis sativa* L. phytocomplex composition [3]. Terpenes represent the largest group of cannabis phytochemicals, with more than 100 identified molecules produced and accumulated in the glandular trichomes abundant on the surface of the female inflorescence [4]. Monoterpenes, diterpenes, triterpenes and sesquiterpenes are important components present in the cannabis resin responsible for its unique aromatic properties. Due to their ability to easily cross cell membranes and the blood-brain barrier, they can also influence the medicinal quality of different cannabis chemotypes [4,5]. At present, there are several incoming and promising applications based on the combined use of cannabinoids and terpenes such as for treatments of acne, sleeping disorders and social anxiety by add caryophyl-

* Corresponding author.
E-mail address: sara.panseri@unimi.it (S. Panseri).

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lene, linalool and myrcene to CBD/THC extracts [6]. As an example, β-caryophyllene selectively binds to the CB2 receptor and could therefore technically be considered as a phytocannabinoid [7]. In addition, differences between the pharmaceutical properties of different cannabis varieties and derived products have been attributed to strictly interactions, defined as 'entourage effect', between cannabinoids and terpenes as a result of synergic action [8]. Medical Cannabis prescriptions are increasing in Italy in line with several other Countries where the therapeutic use is authorised due to its positive role in treating several pathologies [6,9]. As a consequence, Italian galenic pharmacies are authorised to prepare precise cannabis doses for vaping, herbal teas, resins, micronized capsules and oils [9]. This last preparation form received great attention, due to its easy adjustment of the needed individual administration dose along the treatment period, together with the enhanced bioavailability of its active compounds [9].

Very scarce literature is available on the terpene profile in cannabis macerated oil and no information toward their trend during storage [10,11]. In addition, only fragmental investigations on terpenes were conducted using gas-chromatography techniques to describe the volatile fingerprint of medical cannabis chemotypes inflorescences [12]. As recently reviewed by Citti et al. the choice of the employed analytical approach(es) represents today a pivotal task, with particular emphasis on the need for the comprehensive chemical characterisation of the composition of cannabis and derived products [13]. For these reasons, analytical techniques based on GC–MS and HPLC coupled to mass analysers like QTOF and the recently introduced high resolution mass spectrometer HRMS-Orbitrap, represent today the gold standard techniques for the investigation of the highly complex cannabis composition [13].

Based on the above-mentioned considerations, the aims of the present study were:

- To conduct an in-depth fingerprint of volatile compounds, in *C. sativa* L. inflorescences (Bedrocan® and Bediol®) and in their obtained macerated oils during a prolonged storage period (6 weeks), using headspace-solid-phase-microextraction (HS-SPME) and GC–MS.
- To evaluate the feasibility of high resolution mass spectrometry by using LC—HRMS-Orbitrap® for the investigation of targeted and untargeted cannabinoids in Bedrocan® and Bediol® macerated oil during a prolonged storage.
- To set up a new procedure for cannabis macerated oil preparation without any thermal step was tested and compared with the existing conventional methods to assess the potentially detrimental effect of heating on overall product quality.

All analytical methods adopted for the present research were here developed and the applied.

2. Materials and methods

2.1. Chemicals and reagents

All HPLC analytical grade solvents and chemicals were purchased from Sigma (Sigma–Aldrich, St. Louis, MO, USA). Formic acid 98–100% was from Fluka (Sigma–Aldrich, St. Louis, MO, USA). Ultrapure water was obtained through a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). For HS analysis, the SPME coating fibres (polydimethylsiloxane, PDMS, 100 μm; carboxen-polydimethylsiloxane, CAR/PDMS, 75 μm; divinylbenzene-carbowax-polydimethylsiloxane, DVB/CAR/PDMS, 50/30 μm and polydimethylsiloxane, divinylbenzene PDMS/DVB, 65 μm) were from Supelco (Bellefonte, PA, USA). An ultrasonic bath (Branson) was used for cannabis macerated oils preparation (output power 150 W, frequency 35 KHz). A rotary evaporator (Buchi, Swiss) was used to remove organic solvents under reduced pressure. A Retsch high intensity planetary mill (model MM 400, Retsch, GmbH, Retsch-Allee, Haan) was used to obtain representative grinded aliquots of inflorescence samples powder.

2.2. Cannabis plant materials and procedure for oils preparation

Bedrocan® and Bediol® medical Cannabis chemotypes are involved in the present study since they possess a different proportions of cannabinoids (Bedrocan®: 22% THC, <1% CBD; Bediol® 6.5% THC, 8% CBD). Superfine cannabis powder was prepared using mechanical grinding-activation in an energy intensive vibrational mill. Samples were ground in a high intensity planetary mill to obtain a fine powder. The mill was vibrating at a frequency of 25 Hz for 1 min, using two 50 mL jars with 20 mm stainless steel balls. Prior to use, jars were pre-cooled with liquid nitrogen to prevent losses of volatile compounds during grinding process.

Two different extraction procedures were adopted according to previous published studies, while a new additional preparation procedure in which any heating step was omitted has been specifically designed for the present study. The experimental conditions are summarised in Table 1. Regarding method 3 preliminary experiments were done to select the optimum sonication time using THC and CBD as marker analytes (extraction time 30 min Fig. S1). All samples were finally transferred into a 50 mL amber glass bottles to protect oil from light and then stored both at 4 °C or at room temperature (25 °C) for 6 weeks. The bottles were opened and shaken regularly twice daily to simulate the real use conditions. All analyses were done at 0, 7, 14, 21, 28, 35 and 42 days of storage.

2.3. Sample calibration for cannabinoid analysis

Stock solutions (1 mg/mL) of CBDA, CBD, THC, THCA and diazepam-internal standard (IS) in methanol were appropriately

Table 1
Overall medical Cannabis oil's preparation parameters adopted in the present study.

	Oil preparation methods		
	1*	2**	3
Preparation's method references	Romano-Hazekamp [10]	Romano-Hazekamp/Pacifici [10,9]	Present study
Cannabis Flos (Bedrocan-Bediol)	1 g	1 g	1 g
Decarboxylation step	no	Yes/145 °C, 30 min static oven	Yes/145 °C, 30 min static oven
Extraction process	heating in water bath (98 °C 120 min)	heating in water bath (98 °C 60 min)	utrasound (35 KHz 30 min)
Oil heating step	yes	yes	no
Filtration	yes/filter paper	yes/filter paper	yes/filter paper
Final ratio Cannabis/FU oil	1:10	1:10	1:10
Preparation time (min)	150	120	90

* oil procedure according to Romano and Hazekamp, [10].
** oil procedure adopted in Pacifici et al. [9] for decarboxylation step and Romano and Hazekamp, [10] for heating in water bath.

diluted in the oil blank matrices (FU oil) in order to obtain matrix matched calibration curve points with resulting concentrations of 1.0, 5.0, 10.0, 50.0, 100.0 and 1000.0 ng/mL of all compounds and 1 μg/mL of IS. 10 μL of each standard solution were diluted with 890 μL of initial mobile phase, and 2 μL injected in HPLC–MS system.

2.4. Sample preparation

2.4.1. Analysis of volatile compounds from inflorescence and macerated oils

100 mg of *C. sativa* powder or oil were weighed and transferred into 20 mL glass vials and 100 μL of the IS working solution in water for plant samples and in 2-propanol for oils (4-nonylphenol, 2000 μg/mL) was added. Each vial was then fitted with a cap with a silicon/PTFE septum (Supelco, Bellefonte, PA, USA).

2.4.2. Analysis of cannabinoids from macerated oils

The oil samples for HPLC-Q-Exactive-Orbitrap-MS analysis were prepared by dissolving 100 mg of cannabis oil in 10 mL of isopropanol. 1 μg/mL of IS (diazepam) was added and 10 μL of each sample were then added to 890 μL of initial mobile phase from which 2 μL were injected.

2.5. Instruments

2.5.1. HS-SPME

A temperature of 37 °C was selected for both the extraction and equilibration temperature according to previous published research, to prevent possible matrix alterations ensuring the most efficient adsorption of volatile compounds onto the SPME fiber [14,15]. To keep the temperature constant during analysis, the vials were maintained in a cooling block (CTC Analytics, Zwingen, Switzerland). At the end of the sample equilibration time (30 min), a conditioned (60 min at 280 °C) 65 μm Carbowax-divinylbenzene, CAR-PDMS-DVB StableFlex fiber (Supelco, Bellefonte, PA, USA) was exposed to the headspace of the sample for analyte extraction (60 min and 120 min for oil and flos samples respectively) using a CombiPAL system injector autosampler (CTC Analytics, Zwingen, Switzerland).

2.5.2. GC–MS analysis

Analyses were performed with a Trace GC Ultra coupled to a Trace DSQII quadrupole mass spectrometer (MS) (Thermo-Fisher Scientific, Waltham, MA, USA) equipped with an Rtx-Wax column (30 m × 0.25 mm i.d.×0.25 μm film thickness) (Restek, Bellefonte, PA, USA). The oven temperature program was: from 35 °C, held for 8 min, to 60 °C at 4 °C/min, then from 60 °C to 160 °C at 6 °C/min and finally from 160 °C to 200 °C at 20 °C/min. Helium was the carrier gas, at a flow rate of 1 mL/min. Carry over and peaks originating from the fibres were regularly assessed by running blank samples. After each analysis, fibres were immediately thermally desorbed in the GC injector for 5 min at 250 °C to prevent contamination. The MS was operated in electron impact (EI) ionisation mode at 70 eV. A *n*-alkanes mixture (C8-C22, Sigma R 8769, Saint Louis, MO, USA) was run under the same chromatographic conditions to calculate the Kovats retention index (RI) of the detected compounds. The mass spectra were obtained by using a mass selective detector, a multiplier voltage of 1456 V, and by collecting the data at rate of 1 scan/s over the *m*/*z* range of 35–350. Compounds were identified by comparing the retention times of the chromatographic peaks with those of authentic compounds analysed under the same conditions, when available, by comparing the corresponding Kovats retention index with literature data and through the National Institute of Standards and Technology (NIST) MS spectral database (version 2.0.7, 2007). The quantitative evaluation was performed using the internal standard procedure and the results were finally expressed as μg/g or mg/g IS equivalents of each volatile compound. All analyses were done in triplicate.

2.5.3. HPLC-Q-Exactive-Orbitrap-MS analysis

Chromatography was accomplished on an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) that was made up of a Surveyor MS quaternary pump with a degasser, a Surveyor AS autosampler with a column oven and a Rheodyne valve with a 20 μL loop. Analytical separation was carried out using a reverse-phase HPLC column 150 × 2 mm i.d., 4 μm, Synergi Hydro RP, with a 4 × 3 mm i.d. C18 guard column (Phenomenex, Torrance, CA, USA). The mobile phase used in the chromatographic separation consisted of a binary mixture of solvents A (0.1% aqueous formic acid) and B (acetonitrile). The gradient started with 60% eluent A with a linear decrease up to 95% in 10 min. This condition was maintained for 4 min. The mobile phase returned to initial conditions after 14 min, followed by a 6-min re-equilibration period (total run time: 20 min). The flow rate was 0.3 mL/min. The column and sample temperatures were 30 °C and 5 °C, respectively. The mass spectrometer Thermo Q-Exactive Plus (Thermo Scientific, San Jose, CA, USA) was equipped with a heated electrospray ionisation (HESI) source. Capillary temperature and vaporiser temperature were set at 330 °C and 280 °C, respectively, while the electrospray voltage was adjusted at 3.50 kV (operating in both positive and negative mode). Sheath and auxiliary gas were 35 and 15 arbitrary units, with S lens RF level of 60. The mass spectrometer was controlled by the Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA). The exact mass of the compounds was calculated using Qualbrowser in Xcalibur 3.0 software. Instrument calibration was performed at every analytical batch with a direct infusion of a LTQ Velos ESI Positive and Negative Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL, USA). All Q-Exactive parameters (RP, AGC and IT) were optimized to improve sensitivity and selectivity. The FS-dd-MS^2 (full scan data-dependent acquisition) in both positive and negative mode was used for both screening and quantification purposes. Resolving power of FS adjusted on 140.000 FWHM at *m*/*z* 200. For the compounds of interest, a scan range of *m*/*z* 215–500 was chosen; the automatic gain control (AGC) was set at $3e^6$, with an injection time of 200 ms. A targeted MS/MS (dd-MS^2) analysis was performed using the mass inclusion list and expected retention times of the target analytes, with a 10-s time window. This segment operated both in positive and negative mode at 35.000 FWHM (*m*/*z* 200). The AGC target was set to $2e^5$, with the maximum injection time of 100 ms. The precursor ions are filtered by the quadrupole, which operates at an isolation window of *m*/*z* 2. Fragmentation of precursors was optimized as two-stepped normalised collision energy (NCE) (25 and 40 eV) by injected working mix standard solution at a concentration of 100 ng/mL. Detection was based on experimentally calculated exact mass of the protonated/deprotonated molecular ions, at least one corresponding fragment and on retention time of target compounds (Table S3). The mass tolerance window was set to 2 ppm for the two analysis modes. During the preliminary investigation, also parallel reaction monitoring was (PRM) was concerned operative in both positive and negative ionisation at 17.500 FWHM, accompanied with AGC target adjusted on $2e^4$ and the maximum injection time set on 50 ms.

2.6. Methods validation

2.6.1. HS-SPME method optimisation

Optimisation of the method through the selection of SPME fiber coating (PDMS, CAR/PDMS, DVB/CAR/PDMS, PDMS/DVB) together with different extraction times was carried out to select the best

analytical conditions adoptable for the characterization of terpenes profile both from cannabis inflorescence and macerated oil samples. In particular, the four investigated fiber were exposes to the headspace of inflorescences testing four extraction times (30, 60, 120, 180 min) and 3 extraction times (60, 90, 120 min) for oils. 37 °C was selected as the extraction and equilibration temperature according to previously published studies, [14,15].

2.6.2. HPLC-Q-Exactive-Orbitrap-MS method validation

The validation of the HPLC-Q-Exactive-Orbitrap-MS method was performed according to the European Agency for the Evaluation of Medicinal Products (EMEA) analytical techniques for the quality control of pharmaceuticals guidelines. Method validation parameters: stability, specificity, selectivity, linearity, precision, trueness, limit of detection (LOD) and limit of quantification (LOQ). Standard solutions stability dissolved in the mobile phase (acetonitrile: 0.1% formic acid in water, 40:60 v/v) at a concentration of 1 μg/mL was monitored in 5 consecutive days left (storage temperature 5 °C).

Linearity was established using squared correlation coefficients (r^2) on calibration curve points prepared in the matrix.

Trueness was expressed in terms of percentage of measured concentration to nominal concentration ratio at two fortified levels: low (10 ng/mL) and high (1000 ng/mL). The same fortification levels were used for evaluation of method precision. Precision was estimated as the coefficient of variability (CV) for the intra-day and inter-day repeatability. Finally, limits of detection (LODs) and quantification (LOQs) were determined fortifying blank matrix samples at low concentration levels (0.01, 0.05 and 0.1 ng/mL). To specify these lower limits, the LOD was defined as the minimum concentration at which the molecular ion has been detected (mass error < 2 ppm). The arbitrary threshold set at 10.000 was the minimum intensity expected for a possible identification. The LOQ was set up as the minimum concentration where both the molecular ion and either one fragment ion from dd-MS^2 spectrum or corresponding isotope from full mass acquisition have been detected.

2.7. Statistical analysis

All statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, Illinois). Wilcoxon matched pairs test was used for tests on differences of volatile compounds levels among different cannabis inflorescences and oil preparation methods. Significance was accepted at probabilities of 0.05 or less.

3. Results and discussion

3.1. HS-SPME optimisation

3.1.1. HS-SPME fiber selection

In the present study, an HS-SPME procedure was developed for the preconcentration of the volatile compounds present in two commercially available medical cannabis chemotypes and to monitor their stability in oil preparations during storage, with particular focus toward terpenes fraction (mono-, di-, tri-terpenes and sesquiterpenes). Figs. S2 and S3 show the relative area (area of analytes/IS) of total terpenes (sum of mono-di terpenes and sesquiterpenes) extracted by the four tested fibres from cannabis inflorescence and macerated oil. DVB/CAR/PDMS and PDMS/DVB fibres extracted a higher amount of terpenes than the other two tested fibres. On the basis of these results, the DVB/CAR/PDMS fiber was selected.

3.1.2. HS-SPME extraction time

The investigated extraction time varying from 30 to 180 min for plant samples and from 60 to 120 min for macerated cannabis oil (Figs. S2 and S3) were selected based on previous studies on the characterisation of the terpene fingerprint of other officinal plants [14–16]. The highest extraction yields were observed at 60 min and 120 min, considering the overall terpenes profile in the headspace of oil and inflorescence samples respectively.

These results were in accordance with previous studies on the analysis of volatile profile in officinal plants [14–16]. Scarce data are available about the application of HS in the analysis of terpenes and in general of the volatile profile from medical cannabis varieties. Currently, Marchini et al. applied SPME to characterise the terpene profile from hashish, the exudate resin of *C. sativa* L., seeking for potential volatile markers to distinguish the resin from plant material [17].

Volatiles in some Bedrocan® varieties have been previously investigated for their terpenic content by GC-FID, a technique known to furnish only a partial volatile profile, different when compared to that usually emitted by officinal plants [18,19]. In addition, terpenes were extracted using methanol as extraction solvent and then quantified using a calibration curve constructed on the basis of one terpene only. This approach is usually limitative, as the polarity of the solvent could dramatically influence the obtained terpene profile, leading to the underestimation of the complex mixture of secondary metabolites emitted by plants [17].

3.2. HPLC-Q-Exactive-Orbitrap-MS method validation

The overall validation results are summarised in Table 2. Matrix validation curves demonstrated an excellent dynamic range (1–1000 ng/mL) with a good fit ($R^2 > 0.992$) for all compounds. Both precision parameters, inter- and intra-day CV values, were lower than or equal to 15% for all cannabinoids, satisfying the EMEA criteria. Trueness ranged between 79% and 117%, indicating the good efficiency of the preparation protocol. Moreover, standards instability was negligible because the concentrations did not vary significantly (less than 7%).

HPLC–MS/MS techniques has been recently employed for the qualitative and quantitative determination of cannabinoids in plant materials, extracts and biological matrices [5,10].

Optimisation of the HPLC parameters to separate completely the main cannabinoids was done using the reverse-phase HPLC column Synergi Hydro RP (150 mm × 2 mm i.d., 4 μm) and an adequate combination of 0.1% formic acid and acetonitrile as mobile phase. In this context, the particular advantage of HPLC-Q-Exactive-Orbitrap-MS method is that the full scan event in the MS acquisition provides accurate masses for all detected species, thus to allow the post-analysis identification of initially untargeted compounds [20].

As a matter of fact, we were able to detect and to quantify in the macerated oils 7 of the most important cannabinoids, among which 4 essential cannabinoids (THC, CBD, THCA and CBDA) and CBN, CBG and CBGA. In comparison with previous works on cannabis oil [9,11], our HRMS procedure furnished more profound information regarding the cannabinoids profile and amount in the oil composition.

In addition, our method completely overcame previously reported drawbacks of LTQ-Orbitrap–MS, such as slow switching between the positive and negative modes [21]. The majority of previously published MS methods [11] employed only the positive ion acquisition mode as the bioactive, neutral cannabinoids gave an optimal response when registered as protonated pseudo-molecular ions. The fragmentation patterns of our targeted neural cannabinoids were superimposable with those reported in literature data [22].

Chromatograms and corresponding FS and dd-MS^2 spectra are presented in Fig. S4A–C. During preliminary set-up experiments, we noticed that the acidic forms gave from 5 to 20 times better response in negative ionisation mode compared with the positive

Table 2
Validation parameters for HPLC-Q-Exactive-Orbitrap-MS method: calibration curve, correlation coefficients (R^2), limit of detection (LOD), limit of quantification (LOQ), low and high concentration levels, trueness and procedural precision.

Analyte	Calibration curve (n = 3)	R^2	LOD (ng/mL)	LOQ (ng/mL)	Spiked level (ng/mL)	Trueness (%)	Precision (CV; n = 6)	
							intra-day	inter-day
CBD	y = 0,00335(± 0.000053) × + 0,00007 (± 0.000000113)	0.9992	0.01	0.1	10	89	4	10
					1000	91	7	12
THC	y = 0,0024 (± 0.00034) × −0,0029 (± 0.00005)	0.9997	0.01	0.1	10	87	8	9
					1000	101	10	15
CBN	y = 0,0016(± 0.00014) × +0,0006(±0.000009)	0.9973	0.01	0.1	10	79	7	6
					1000	107	8	10
CBG	y = 0,0018(± 0.00034) × +0,0182 (±0.00114)	0.9991	0.01	0.1	10	101	7	11
					1000	89	11	12
CBD-A	y = 0,0231(±0.0006) × +0,0247(±0.00047)	0.9985	0.01	0.05	10	95	6	8
					1000	107	8	10
THC-A	y = 0,0055(±0.000017) × +0,0091(±0.00016)	0.9913	0.01	0.05	10	92	10	11
					1000	89	5	8
CBG-A	y = 0,0157(±0.000273) × −0,0052(±0.00012)	0.9997	0.01	0.05	10	104	6	12
					1000	111	5	9

one (Fig. S4D–E). Information about the negative fragmentation pattern of main acidic cannabinoids forms is very limited in the available literature. The THCA fragmentation in negative ion mode corresponded only to one published [23], while those for CBDA and CBGA has not been previously reported in details.

Both THCA and CBDA showed a molecular ion at *m*/*z* 357 and although both presenting the same fragments, they differed notably regarding the intensities of two most characteristic signals, namely *m*/*z* 313 and *m*/*z* 339. The deprotonated pseudo-molecular ion subsequently loses either the carboxylic group producing a fragment ion of *m*/*z* 313, or one hydroxyl group leading to the formation of the ion at *m*/*z* 339. Neutral loss of C_5H_8 and opening of the A ring from the decarboxylated produce the *m*/*z* 245 fragment, from witch an additional α-cleavage of the pentyl side chain gave a *m*/*z* 191 fragment ion.

3.3. Applications to medical cannabis

3.3.1. Characterisation of terpenes profile of Bedrocan® and Bediol®

The volatile profiles extracted using the optimized HS-SPME and GC–MS method from *Bedrocan® and Bediol®* were presented and summarised in Table 3. Overall, up to 100 volatile compounds composed their volatile fingerprints of which 72 identified terpenes. The differences between individual representative terpenes (>80% total terpenes) are presented in Fig. S5.

Bedrocan® was richer in terpene content compared to Bediol®. In particular, the representative terpenes α-pinene and β-pinene, α-phellandrene and α-terpinolene were detected in higher concentration in Bedrocan® as shown in Table 3. By contrast, β-myrcene was higher in Bedio®l chemotype. The latter was also richer in esters, volatile compounds responsible and associated with "fruity" flavour notes.

In comparison with cannabinoid derivatives, the volatile constituents of *C. sativa* L. received much less attention [2,24,8]. Nevertheless, the chemical composition of cannabis essential oils is well documented and one study investigated the volatile profile of some cannabis varieties from central Asia [3]. At present, scarce emphasis was given toward the exhaustive characterisation of the terpenes profile in cannabis chemotype standardised and certified for medical uses.

In relation to recent evidence for the synergic action of terpenes and cannabinoids (entourage effect), the comprehensive evaluation of terpene compounds, especially those characterising medical chemotypes, is today crucial for the management of cannabis as complete therapeutic tool and to select the most appropriate chemotype for therapeutic use.

Beside hydrocarbon terpenes, also oxygenated terpenoids such as linalool, verbenol, terpinen-4-ol and alfa-terpineol, alcohols, aldehyde and ketones were detected (Table 3). Most of these oxygenated compounds corresponded to secondary photo-oxidation's products of the initial terpenes. In presence of light and singlet oxygen, terpenes are known to undergo photo-oxidation leading to the formation of allylic hydroperoxides [17]. Since hydroperoxides are generally unstable, they will generate the corresponding alcohols that may be further oxidized to the corresponding aldehydes and ketones [17], e.g. the degradation of limonene that under photo-oxidation leads to the formation of *trans*- and *cis*-metha-2,8-dien-1-ol and *trans*- and *cis*-carveol. Under the same mechanism, β-myrcene, that strongly characterise some medical cannabis varieties, can be transformed into ipsdienol and 2-methyl-6-methylene-octa-3,7-dien-3-ol. Hence, the HS-SPME approach provides the advantage to determine and to evaluate also oxidation phenomena especially occurring during inflorescence storage and processing.

Table 3
Volatile compounds profile extracted by using HS-SPME and GC–MS from two different medical cannabis varieties (Bedrocan and Bediol)

RT[a]	RI[b]	Compound	Bedrocan		Bediol		F[e]	p-value	Signif. Code[f]
			Mean[c]	±SD[d]	Mean	±SD			
Alcohols									
19.81	746	3-Methyl-2-buten-1-ol	9.11	2.38	n.d.	–	43.41	0.0027	**
20.69	831	1-Hexanol	318.14	15.51	393.48	46.97	4.13	0.1119	ns
21.48	868	3-Hexen-1-ol	18.80	1.05	25.81	3.20	0.32	0.6007	ns
27.96	1068	3,3,6-Trimethyl-1,5-heptadien-4-ol	n.d.	–	445.50	33.32	513.75	0.0224	***
31.63	1036	α-Toluenol	28.30	7.50	9.17	1.34	20.25	0.0108	*
32.09	1136	Benzeneethanol	55.23	5.06	24.22	2.26	83.61	0.0008	***
		tot	429.58		898.19				
Aldehydes									
3.75	643	2-Methyl-butanal	4.21	2.11	3.07	0.46	29.65	0.1602	ns
10.08	785	Hexanal	28.01	9.44	35.91	15.11	0.11	0.7517	ns
24.78	982	Benzaldehyde	195.08	87.26	256.35	14.65	0.12	0.7418	ns
		tot	227.30		295.33				
Ketones									
20.21	987	6-Methyl-5-hepten-2 one	20.22	2.93	28.92	1.20	0.01	0.9160	ns
22.51	1012	Ketone	n.d.	–	20.76	0.54	228.25	0.0001	***
33.99	1250	Ketone	13.85	2.15	6.11	0.66	67.96	0.0012	**
		tot	34.07		55.79				
Esters									
2.63	487	Acetic acid-methyl ester	153.85	57.52	99.65	18.85	64.18	0.0644	.
5.70	686	Butanoic acid-methyl ester	85.12	44.81	96.41	16.45	0.43	0.5476	ns
15.21	884	Hexanoic acid-methyl ester	n.d.	–	492.79	64.58	34.16	0.1383	ns
17.85	892	3-Hexenoic acid-methyl ester	n.d.	–	34.99	11.72	18.82	0.0123	*
20.29	1083	Propanoic acid-hexyl ester	49.05	2.02	69.46	1.78	0.17	0.7039	ns
20.37	1118	Propanoic acid-2-methyl-hexyl ester	128.35	6.70	132.25	2.84	42.51	0.0029	**
22.30	1183	Butanoic acid-hexyl ester	1076.28	76.79	844.92	17.87	140.30	0.0003	***
24.05	1210	Nonanoic acid-methyl ester	10.61	2.40	19.28	4.25	24.63	0.1916	ns
26.67	1381	Hexanoic acid-hexyl ester	69.95	4.77	321.76	38.61	654.96	0.0139	***
31.85	1550	Butanoic acid-3,7-dimethyl-2,6-octadienyl ester	14.56	0.85	n.d.	–	4389.00	0.0000	***
35.91	1372	Benzoic acid-2-amino-methyl ester	69.15	17.27	127.22	1.27	3.23	0.1465	ns
		tot	1656.91		2238.72				
Mono-di-tri Terpenes									
6.78	939	α-Pinene	2114.60	330.37	2522.22	220.54	31.22	0.1520	ns
7.09	932	α-Thujene	485.72	89.40	73.97	11.62	87.97	0.0007	***
8.36	943	α-Fenchene	33.40	7.91	n.d.	–	61.82	0.0014	**
8.71	961	Camphene	145.12	29.28	49.59	8.55	42.12	0.0029	**
10.54	989	β-Pinene	3261.72	957.34	1369.80	129.52	22.05	0.0093	**
11.28	985	Sabinene	110.42	58.64	15.56	4.68	93.60	0.0377	*
11.72	1349	Terpene	45.81	16.69	n.d.	–	26.11	0.0069	**
12.68	1017	δ-3-Carene	2325.94	708.58	361.47	65.98	33.52	0.0044	**
13.60	1015	α-Phellandrene	2708.02	1008.29	345.51	84.60	21.99	0.0094	**
14.26	991	β-Myrcene	14827.96	2033.32	27691.29	4039.57	78.98	0.0483	*
14.46	1026	α-Terpinene	1876.40	338.44	406.33	112.23	108.85	0.0005	***
15.27	1038	Limonene	4860.14	1228.58	930.57	67.23	52.24	0.0019	**
15.50	1045	Eucalyptol	n.d.	–	73.94	0.68	38.37	0.1217	ns
15.58	946	β-Phellandrene	3621.50	1175.70	1330.19	158.76	19.70	0.0113	*
17.00	976	Cis-ocimene	264.70	57.16	93.67	11.23	45.43	0.0025	**
17.12	1066	γ-Terpinene	1237.25	620.12	458.34	55.09	74.64	0.0523	.
17.70	1029	β-Ocimene	9987.94	640.36	2426.19	87.96	2149.80	0.0013	***
18.03	1034	p-Cymene	858.37	165.14	330.38	38.82	49.33	0.0022	**
18.76	1094	α-Terpinolene	35004.04	3278.32	8924.58	653.45	1105.80	0.0049	***
19.99	1121	Terpene	16.29	2.20	n.d.	–	207.96	0.0001	***
21.08	1029	1,3,8-*p*-Menthatriene	8.05	0.92	n.d.	–	336.35	0.0001	***
21.72	993	Alloocimene	66.56	10.83	14.08	0.60	90.39	0.0007	***
22.07	1013	Cis-epoxy-ocimene	n.d.	–	22.19	1.07	549.63	0.0196	***
22.41	1039	Terpene	151.65	26.71	100.84	6.26	33.11	0.0045	**
22.78	1073	p-Cymenyl	1158.87	99.86	543.24	8.60	263.25	0.0844	***
23.04	1035	Terpene	235.00	9.08	67.90	2.45	2739.20	0.0008	***
23.39	1083	4,8-Epoxy-*p*-menth-1-ene	566.84	17.56	227.75	16.17	1371.80	0.0032	***
25.34	1082	β-Linalool	557.91	93.52	41.34	2.80	138.83	0.0003	***
26.40	1193	4-Terpineol	n.d.	–	108.96	10.15	423.82	0.0329	***
26.89	1136	Verbenol	n.d.	–	149.20	10.15	955.13	0.0065	***
28.37	1209	α-Terpineol	418.76	40.67	169.04	20.70	303.99	0.0635	***
30.04	1211	γ-Geraniol	n.d.	–	17.94	3.56	219.33	0.0001	***
30.44	1042	β-Cymene	54.20	5.56	85.49	2.27	12.27	0.3302	ns
31.21	1284	Cuminol	440.92	11.16	245.62	15.45	271.44	0.0795	***
31.41	1158	β-Terpineol	n.d.	–	14.43	2.77	228.51	0.0001	***

Table 3 (*Continued*)

RT[a]	RI[b]	Compound	Bedrocan		Bediol		F^e	*p*-value	Signif. Code[f]
			Mean[c]	±SD[d]	Mean	±SD			
34.86	1384	Terpene	n.d.	–	17.51	2.48	47.79	0.0023	**
		tot	87444.07		49229.13				
Sesquiterpenes									
23.18	1344	α-Cubebene	84.12	7.25	11.58	2.25	205.93	0.0001	***
23.75	1221	α-Ylangene	354.60	15.44	45.39	7.97	357.67	0.0460	***
23.95	1430	α-Copaene	170.08	7.13	52.11	3.48	225.10	0.0001	***
25.06	1451	Zingiberene	127.10	10.14	51.26	9.63	91.06	0.0007	***
25.42	1474	Sesquiterpene	514.57	77.42	125.66	4.04	76.40	0.0009	***
25.55	1393	Sesquiterpene	137.35	9.91	84.07	5.37	71.23	0.0011	**
25.80	1493	Sesquiterepene	225.71	14.34	189.42	5.60	159.72	0.0002	***
25.87	1416	Sesquiterpene	468.84	34.30	224.95	22.74	112.96	0.0004	***
26.19	1430	α-Bergamotene	1321.65	64.04	1025.66	67.87	68.39	0.0012	**
26.29	1456	α-Guaiene	852.56	155.16	1354.45	81.83	11.46	0.3447	ns
26.35	1494	Trans-caryophyllene	3335.85	211.33	3070.13	322.32	107.78	0.0005	***
26.54	1403	Aristolene	150.67	10.19	n.d.	–	231.37	0.0001	***
27.04	1419	Isoledene	113.35	10.04	11.58	1.64	150.03	0.0003	***
27.12	1425	β-Santalene	31.81	2.23	24.34	2.08	42.46	0.0029	**
27.22	1386	Aromadendrene	239.26	14.09	70.36	6.86	182.34	0.0002	***
27.32	1407	Sesquiterpene	n.d.	–	10.26	2.27	140.22	0.0003	***
27.45	1446	β-Sesquiphellandrene	n.d.	–	35.49	4.99	1747.40	0.0020	***
27.73	1474	Sesquiterpene	113.46	2.70	2230.02	171.99	2087.10	0.0014	***
27.84	1482	α-Humulene	3078.46	60.00	n.d.	–	744.22	0.0107	***
27.88	1502	γ-Selinene	261.95	3.96	220.26	31.77	101.54	0.0005	***
28.03	1387	Sesquiterpene	125.69	1.04	n.d.	–	1450.70	0.0028	***
28.12	1440	β-Farnesene	91.77	3.43	81.64	9.94	95.57	0.0006	***
28.23	1502	Sesquiterpene	797.66	10.03	171.43	7.54	992.88	0.0060	***
28.57	1398	Sesquiterpene	133.69	3.50	128.41	15.23	77.59	0.0009	***
28.78	1490	δ-Guaiene	1409.25	238.01	1470.24	137.71	79.45	0.0479	*
28.91	1522	α-Selinene	783.22	11.87	679.33	65.01	244.50	0.0977	***
29.07	1419	Sesquiterpene	1132.64	39.58	338.46	37.06	424.36	0.0000	***
29.25	1497	γ-Curcumene	59.17	4.39	105.29	7.81	83.97	0.0442	*
29.43	1458	α-Farnesene	918.96	71.06	608.18	76.89	67.37	0.0012	**
29.49	1556	δ-Cadinene	213.11	6.09	110.67	15.78	447.73	0.0295	***
29.57	1416	α-Panasinsen	n.d.	–	41.62	4.09	7450.40	0.0000	***
29.64	1411	Sesquiterpene	64.24	3.95	n.d.	–	3100.00	0.0000	***
29.79	1432	β-Maaliene	1232.11	27.74	111.25	15.93	738.25	0.0109	***
29.87	1518	Sesquiterpene	142.85	13.43	n.d.	–	149.46	0.0003	***
30.02	1507	Selina-3,7(11)-diene	4419.91	224.49	2038.83	222.68	195.69	0.0002	***
30.20	1443	α-Muurolene	17.53	1.29	16.32	2.41	25.79	0.0071	**
30.36	1461	γ-Gurjunene	73.61	11.82	n.d.	–	86.88	0.0007	***
30.80	1481	Sesquiterpene	288.46	13.97	n.d.	–	298.79	0.0657	***
30.87	1603	Germacrene	376.52	56.83	n.d.	–	98.29	0.0006	***
32.15	1547	α-Calacorene	19.88	1.97	26.71	5.98	0.29	0.6187	ns
32.88	1617	Caryophyllene oxide	39.58	3.57	34.11	6.42	40.25	0.0032	**
33.40	1564	Nerolidol	59.33	1.56	89.69	14.93	0.44	0.5428	ns
		tot	23980.56		14889.17				
Miscellaneous									
2.05	550	Dimethyl sulfide	3.00	1.63	2.02	0.39	29.96	0.1585	ns
2.41	788	2,4-Dimethyl-heptane	n.d.	–	1.64	0.20	76.62	0.0009	***
2.90	852	4-Methyl-octane	n.d.	–	3.34	0.28	115.15	0.0004	***
4.71	742	2-Ethyl-furan	n.d.	–	2.44	0.09	201.43	0.0001	***
16.89	1040	2-Pentyl-furan	n.d.	–	7.86	4.13	87.56	0.0416	*
21.29	1006	1-Ethyl-3-methyl-benzene	12.55	1.31	n.d.	–	888.14	0.0000	***
21.63	1013	(2-Methylprop-1-enyl)-cyclohexa-1,3-diene	107.63	12.27	42.06	1.66	156.86	0.0002	***
32.32	1138	Benzyl nitrile	43.38	0.79	21.87	1.22	618.23	0.0155	***
		tot	166.57		81.23				

RT[a]: retention time (min) RI[b]: retention index calculated on a Rtx-Wax (30 m x 0,25 mm x 0,25 μm f.t.) Mean[c]: Mean value (n = 3); Data are expressed in ppm SD[d]: Standard deviation (n = 3); F[e]: F-statistics Signif. Code[f]: *** = 0.0001; ** = 0.001; * = 0.01; – = 0.05; ns = not significant

3.3.2. Evolution and stability of terpenes, secondary lipid's oxidation products and cannabinoids of Bedrocan® and Bediol® oil preparations during storage

Overall data concerning cannabinoid contents, terpenes and lipid oxidation products extracted from the three tested oil preparation methods and their trend during a 6-weeks storage are summarised and reported in Figs. 1–6 and table S4-S7.

3.3.2.1. Terpenes and secondary lipid's oxidation products of Bedrocan® and Bediol® oil preparations during storage. Concerning Bedrocan® -based oils, 45 terpenes were determined using SPME extraction methods in all preparations and the most abundant were β-myrcene, β-pinene, D-limonene, α-terpinene, α-terpinolene, δ-3-carene, β −ocimene and β-caryophyllene. At present only Romano and Hazekamp analysed some terpenes by using GC-FID, reporting the identification of only 12 terpenes from Bedrocan oil with not quantitative information since the results were expressed as arbitrary units.

Comparing the three different preparation methods, it can be observed that method 1 was capable to extract the highest amount of terpenes, followed by method 3 and 2 (Fig. 1 $p < 0.01$).

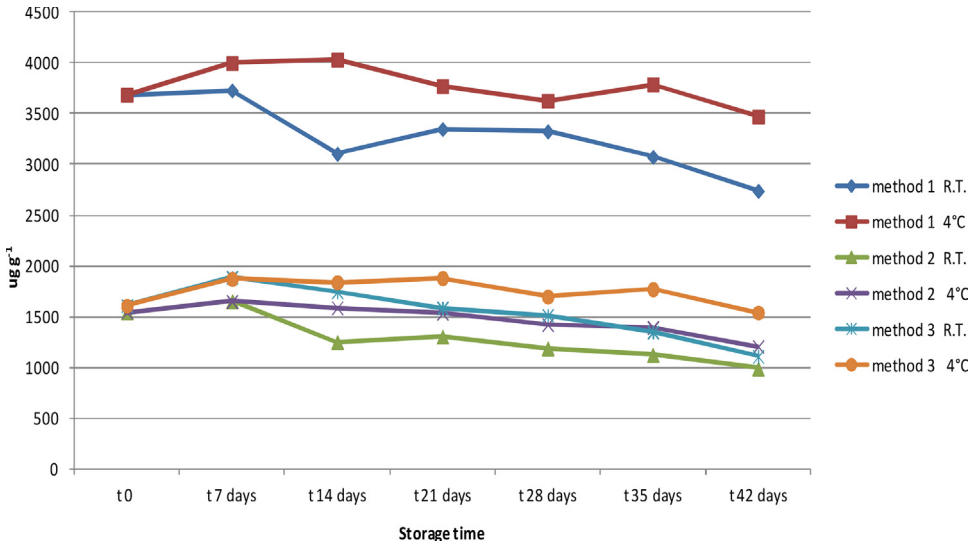

Fig. 1. Bedrocan's terpenes trends during storage at room temperature and at 4 °C in different cannabis oils (sum of mono-di-tri and sesquiterpenes).
* = Data are expressed as $\mu g\ g^{-1}$; method 1 = oil procedure according to Romano and Hazekamp, [10]; method 2 = oil procedure adopted in Pacifici et al. [9] for decarboxylation step and Romano and Hazekamp, 2013 for heating in water bath; method 3 = oil procedure optimized in the present study based on ultrasound extraction. Mean standard deviation is reported in the respective tables

However, the decarboxylation step is often necessary to generate the bioactive CBD and THC.

The time- and temperature-dependent trends observed during storage showed significant differences. In particular, at 4 °C a minor decrease in terpenes concentrations was found ($P < 0.05$).

It was also useful to compare method 3 and 2 since the corresponding decarboxylation steps were conducted at the same temperature (145 °C, 30 min). The macerated oil obtained with method 3 (no heating step) showed a higher concentration of terpenes (Fig. 1).

Examining the terpenes reduction percentages by comparing terpene content at final storage time (42 days) to the starting content, we observed minor decreases in oils obtained with method 2 and 3 (4 °C; 5.50% and 4.92%, respectively) than with method 1, in which a decrease of 15.32% was calculated.

Considering lipid oxidation products, the opposite trend was shown among the three preparation procedures. As a consequence of storage temperatures, the highest concentrations of ketones and aldehydes were for samples stored at room temperature compared to refrigerated conditions. In particular, method 3 realised without any heating step, showed minor concentrations of lipid oxidation products throughout the entire storage period (Fig. 2).

The macerated oil obtained using the method by Romano-Hazekamp (method 1) contained the highest levels of oxidation products, compared with the other two procedures as expected. This can be related to preparation conditions in which the oil is heated at 98 °C for 120 min.

No data concerning lipid oxidation products formation during cannabis oil maceration at different temperatures and maceration times has been previously reported. It has been previously demon-

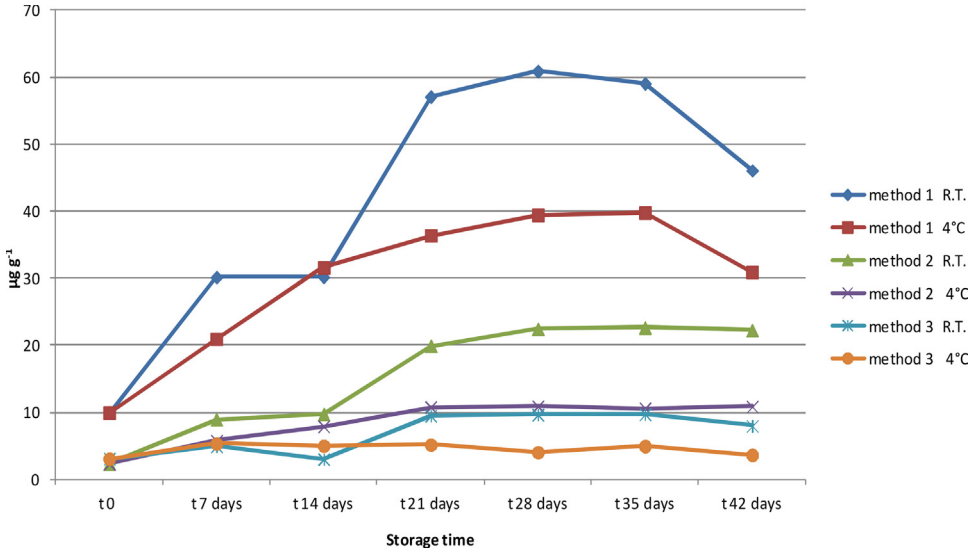

Fig. 2. Bedrocan's lipid oxidation compounds trends during storage at room temperature and at 4 °C in different cannabis oils (sum of mono-di-tri and sesquiterpenes).
* = Data are expressed as $\mu g\ g^{-1}$; method 1 = oil procedure according to Romano and Hazekamp, [10]; method 2 = oil procedure adopted in Pacifici et al. [9] for decarboxylation step and Romano and Hazekamp, [10] for heating in water bath; method 3 = oil procedure optimized in the present study based on ultrasound extraction. Mean standard deviation is reported in the respective tables

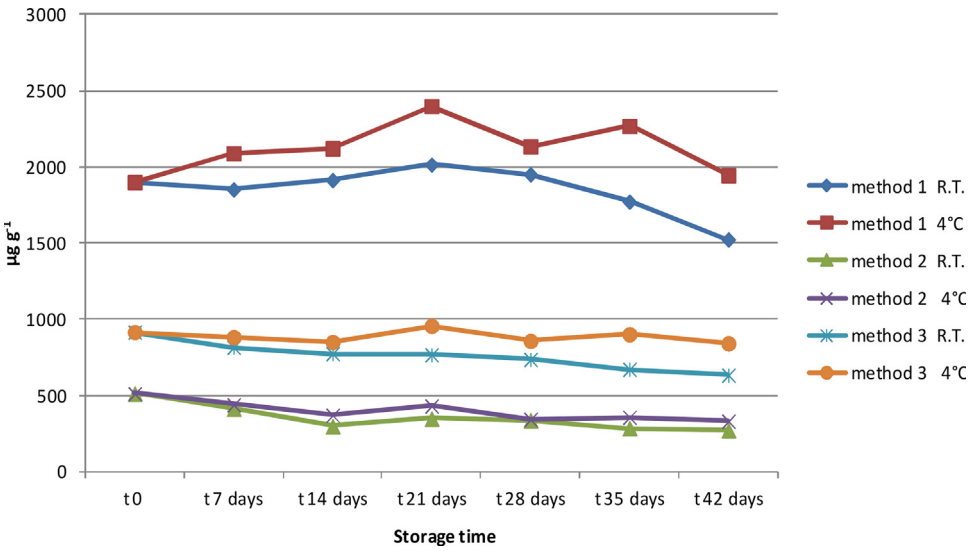

Fig. 3. Bediol's terpenes trends during storage at room temperature and at 4 °C in different cannabis oils (sum of mono-di-tri and sesquiterpenes).
* = Data are expressed as $\mu g\,g^{-1}$; method 1 = oil procedure according to Romano and Hazekamp, [10]; method 2 = oil procedure adopted in Pacifici et al. [9] for decarboxylation step and Romano and Hazekamp, [10] for heating in water bath; method 3 = oil procedure optimized in the present study based on ultrasound extraction. Mean standard deviation is reported in the respective tables

strated that heating can mediate the formation of several "*ex-novo*" lipids breakdown products (mainly ketones and aldehydes), that can strongly influence the oil digestibility and stability [25]. This is particular evident for foods rich of unsaturated fatty acids like olive oil (Panseri et al.) [25]. Examining the Bediol® -based oils, compared to Bedrocan® -based oil the same trend was observed and presented in Fig. 3 and 4.

47 terpenes were extracted and identified and the most abundant were: α-pinene, β-pinene, β-myrcene, α-terpinolene, β-ocimene and β-caryophyllene.

Examining the terpenes reduction percentages by comparing terpene content at final storage time (42 days) to the initial content, we observed the minor decreasing in method 1 and 3 stored a 4 °C (8.80% and 18.35%, respectively) than method 2 in which a 35.32% decrease was calculated. As also evidenced in Bedrocan® oil shelf-life, evaluating the lipid oxidation products formed during storage time, the major concentration was found in method 1 stored at room temperature, in which thermal step of 120 min were adopted to extract terpenes and cannabinoids from oil. Very scares information are available in literature dealing to the study of terpenes in cannabis oils. Only Citti et al. characterised some terpenes content by using a destructive technique in cannabis oil but no evaluation was then made concerning their trend and stability during storage [11].

3.3.2.2. Cannabinoids trend in Bedrocan® and Bediol® oil preparations during storage. The overall data of target cannabinoids investigated during Bedrocan® and Bediol® macerated oils storage are presented in Figs. 5 and 6.

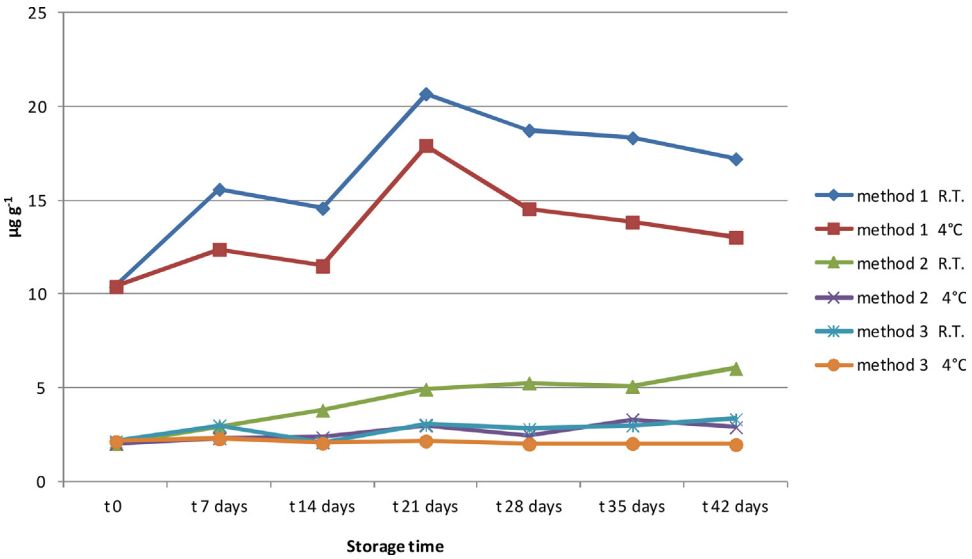

Fig. 4. Bediol's lipid oxidation compounds trend during storage at room temperature and at 4 °C in different cannabis oils (sum of mono-di-tri and sesquiterpenes).
* = Data are expressed as $\mu g\,g^{-1}$; method 1 = oil procedure according to Romano and Hazekamp, [10]; method 2 = oil procedure adopted in Pacifici et al. [9] for decarboxylation step and Romano and Hazekamp, [10] for heating in water bath; method 3 = oil procedure optimized in the present study based on ultrasound extraction. Mean standard deviation is reported in the respective tables

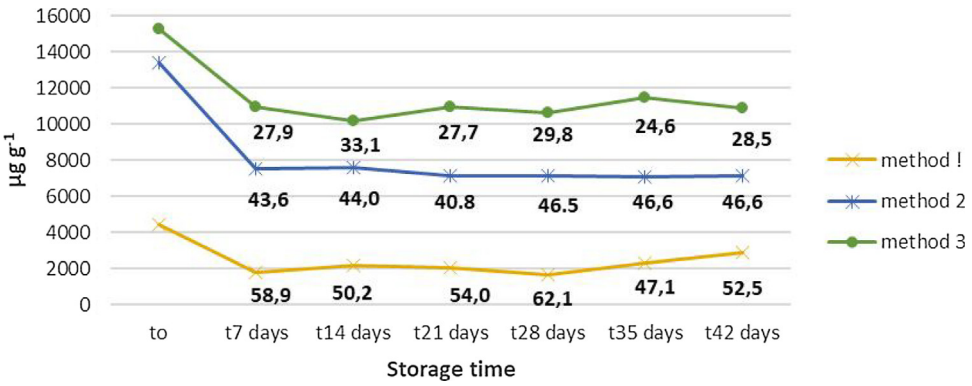

Fig. 5. Bedrocan's THC trends during storage at 4 °C in different cannabis oil preparation methods.
* = Data are expressed as µg g^{-1}; method 1 = oil procedure according to Romano and Hazekamp, [10]; method 2 = oil procedure adopted in Pacifici et al. [9] for decarboxylation step and Romano and Hazekamp, [10] for heating in water bath; method 3 = oil procedure optimized in the present study based on ultrasound extraction. Numbers indicates the percentage reduction of cannabinoids content during storage compared to the initial content.

Method 3 (preheating/ultrasounds assisted extraction), showed the highest extraction yields of the neutral cannabinoids CBD and THC. By contrast, method 1 furnished the maximal concentrations of THCA, CBDA and CBGA.

At present, it is important to emphasize that, in the ambit of the therapeutic uses of cannabinoids related to pharmacological and clinical effects, THC and CBD in their neutral forms are of primary interest, even if there is growing attention toward the acidic forms also [26,27].

Considering the decrease of THC and CBD concentrations observed in Bediol® and Bedrocan® during 6-weeks of storage, the minor reduction was found in preparation 3 for both the two investigated medical chemotypes, as reported in Fig. 5 and 6.

In agreement with previous studies [9,11] on Bediol® and FM2 chemotype macerated oils, the major reduction of cannabinoids occurred during the first week of storage, even if no other study have investigated a long shelf-life (up to 15 days) until the present research. The data obtained considering a prolonged storage period are useful considering that the common medical prescriptions are usually prepared for 30 days of treatment.

Comparing the results obtained under refrigeration and at room temperature, no significant differences were observed for the main cannabinoids concentrations, although at some time-points, storage at room temperature gave slightly higher values. Most probably, this can be attributed to the physicochemical characteristics of oil itself, which undergoes a liquid to semi-solid consistency transition at low temperature, requiring its liquefaction before analysis. It is likely that this phenomenon contributed to a higher heterogeneity of the matrix and had a decisive effect on the distribution of cannabinoids in the oil matrix.

An alternative or concomitant explanation for these unusual variations can be the heat-mediated generation of hydroperoxides that along storage can contribute to a partial cannabinoids oxidization. Conversely, the low but detectable instability of cannabinoids

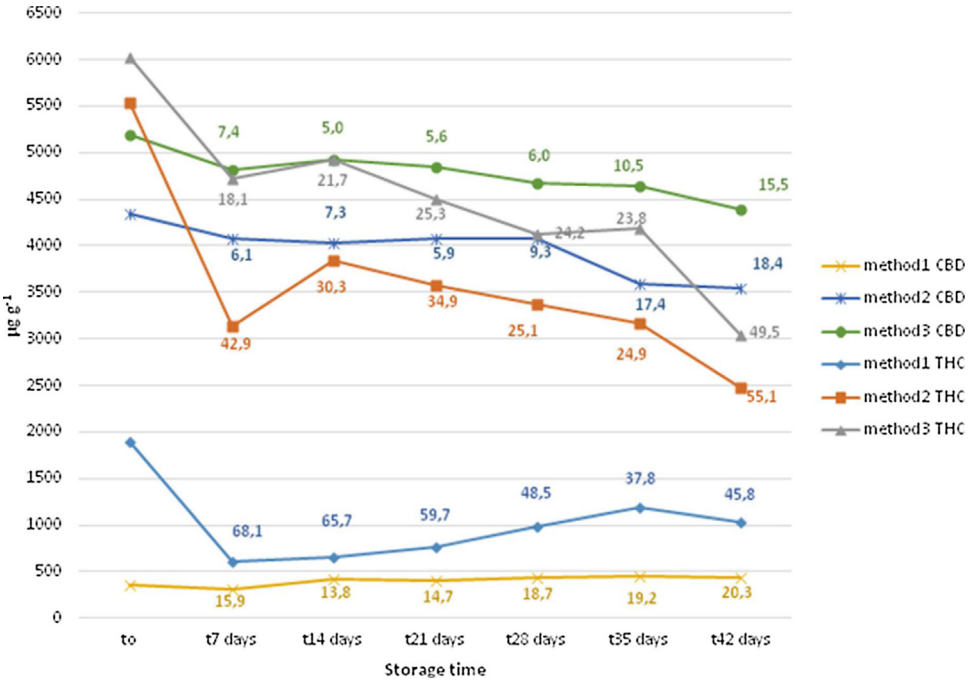

Fig. 6. Bediol's THC and CBD trends during storage at 4 °C in different cannabis oil preparation methods.
* = Data are expressed as µg g^{-1}; method 1 = oil procedure according to Romano and Hazekamp, [10]; method 2 = oil procedure adopted in Pacifici et al. [9] for decarboxylation step and Romano and Hazekamp, [10] for heating in water bath; method 3 = oil procedure optimized in the present study based on ultrasound extraction. Numbers indicates the percentage reduction of cannabinoids content during storage compared to the initial content.

in cannabis macerated oils reported by Citti et al. can be explained by hydroperoxides degradation induced by the higher temperature applied for extraction [11]. Further studies are needed to clarify this point.

Besides the principal targeted compounds, several other minor "untargeted" compounds were detected by means of Orbitrap® analyser. Their structural elucidation was achieved from the mass spectra collected in the FS and corresponding dd-MS^2 scan modes, and relied on the information found in the literature [4,5].

Among untargeted molecules, we verified the presence of THC-C4, CBD-C4, THCV-C3, CBD-C3 which expressed the same fragmentation behaviour as their C5 equivalents but differed in fragments that contained the C3 or C4 side chain (Fig. S6). Furthermore, one of the important untargeted acid was screened, namely CBNA. The FS and dd-MS^2 identification was accomplished in negative mode as for all acids (Fig. S7). The unexpected, rather high intensity of CBNA suggests that it would be useful to perform also its quantification in future studies. CBNA follow up could be useful as a marker of THCA transformation during the storage, especially when the decarboxylation step during oil preparation is omitted (method 1).

In addition, two THC structural isomers with the same nominal mass and molecular formula and different retention times were observed. Most presumably, these peaks were generated by cannabichromene (CBC) and cannabicyclol (CBC), but their signals were too weak to trigger the specific fragmentation in dd acquisition mode.

The CBN peak was visible and quantifiable in all the real samples investigated, but in those obtained by extraction method 3, another peak (RT = 11.65 min) with the same nominal mass was noticed. The fragmentation pattern was similar to that of CBN, with an additional fragment at m/z 270.27899 (Fig. S8). This suggested a cannabifuran structure (CBF-C5), as the furan ring opening subsequent intramolecular arrangements could form the above mention fragment.

During initial set-up of instrumental method, we estimated that dd-MS^2 gave the better confirmation approach for the presence of untargeted minor cannabinoids compared with PRM which is equivalent to triple quadrupole functioning. Both dd-MS^2 and PRM gave the comparable absolute response, but dd-MS^2 mode was much more specific. As an example in Fig. S9 dd-MS2 and PRM spectra are compared for the same real Bedrocan® oil sample regarding the presence of untargeted THC-C4. It remains also to be established weather the cannabinoids such as CBN and CBNA, occurring as artefacts during the degradation/oxidation of mainly THC and THCA can be used as suitable markers of cannabinoids stability in cannabis macerated oil preparations.

One last aspect to take into consideration in order to evaluate the benefits of various extraction methods is the extent to which various procedures are safe and time-consuming both in the laboratory and in galenic pharmacies. Method 3 reduces dramatically the preparation time, introducing safer working conditions as no thermal disposal are is necessary during oil extraction (Table 1).

4. Conclusions

In the present study, an analytical procedure involving the combination of HS-SPME coupled to GC–MS and HPLC-HRMS (Orbitrap®) was set up, validated and applied for the in-depth profiling and fingerprinting of cannabinoids and terpenes in two authorised medical grade varieties of *Cannabis sativa* L. *flos*. (Bedrocan® and Bediol®) and their macerated oils.

HS-SPME represents an excellent technique to investigate both the cannabis inflorescence and derived macerated oil volatile fractions. In particular, HS extraction allowed to obtain accurate information about their terpenes fingerprints. The exhaustive comprehension of terpenes profile today represent a pivotal approach since they are today studied for their synergic and therapeutic role if combined to cannabinoids in an "entourage effect".

LC-HRMS-Orbitrap® used to investigated cannabinoids extracted from oils possesses high-throughput performances, as it is able to be used both for quantification of target analytes at very low concentrations and to investigate also the untargeted fraction to obtain a very complex prolife as an expression of plant phytocomplex at the same time.

Concerning the cannabis macerated oil preparations, the new method realised by ultrasound extraction without any heating step during maceration, allowed to obtain the maximal concentrations of CBD and THC in the final extract, thus preserving cannabinoids and terpenes, since the minor reduction percentages were shown especially in the last storage days. In addition, it can easily realised from galenic pharmacies that are involved in the preparation of *C. sativa* derived-products as oils for medical uses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jpba.2017.11.073.

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