Medicinal *Cannabis*: extended stability of *Cannabis* extracts produced using a new ethanol-based extraction method

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Abstract

Cannabis as a therapeutic agent is increasing its popularity all around the globe, particularly in Western Countries, and its potential is now well assessed. On the other hand, each Country has its own regulation for the preparation of *Cannabis* macerated oils, in Italy there are only few preparation methods allowed. With this work we aim to perform a stability study of *Cannabis* oils produced with a novel method for the extraction of cannabinoids from *Cannabis* inflorescence. Three different varieties of *Cannabis* were used, with and without the adding of tocopherol acetate as an antioxidant. Cannabinoids were extracted using ethanol at room temperature, then the solvent was evaporated under reduced pressure and the preparations reconstituted with olive oil. In this work, we assessed the stability of both cannabinoids and terpenes in these formulas along 8 months. Cannabinoids stability was assessed monitoring the concentrations of THC and CBD, while terpenes stability was assessed monitoring β-Caryophyllene and α-Humulene concentrations. Stability of the extracts was not influenced by the presence of tocopherol acetate, though refrigeration seems to be detrimental for a long storage of products, especially regarding THC concentrations. The improvements offered by this method resides in the flexibility in controlling the concentration of the extract and ability to produce highly concentrated oils, alongisde the possibility to produce standardized oils despite the variability of the starting plant material.

Keywords: *Cannabis*, Cannabaceae, stability, macerated oils, extraction, galenic formulas

List of Abbreviations: CBD, cannabidiol; CBDA, cannabidiolic acid; SHS, static headspace sampling; SIFAP, Società Italiana Farmacisti Preparatori; THC, delta-9 tetrahydrocannabinol; THCA, delta-9 tetrahydrocannabinolic acid.

Introduction

The use of *Cannabis* in clinical settings is now well-established as a treatment for different pathological conditions, such as anorexia, drug-induced nausea, multiple sclerosis and symptoms associated with HIV/AIDS [1–3]. The major chemical constituents in *Cannabis* metabolic profile are the cannabinoids. Cannabinoids are terpenophenolic compounds that are believed to be directly responsible for the plant's pharmacological effects. There are more than one hundred identified cannabinoids and they can be found in all the aerial parts of the plant but are mostly concentrated in the female flowers.

Especially two cannabinoids are considered accountable for both psychoactive and therapeutic effects and therefore dosed for medications, namely delta-9 tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA); it is worth noticing though that both THCA and CBDA become pharmacologically active after decarboxylation to delta-9 tetrahydrocannabinol (THC) and cannabidiol (CBD) [4]. In Italy, different varieties of *Cannabis* are approved for therapeutical purposes, according to the titrated concentration of psychoactive substances in the plant, for example Bedrocan (THC=17.6-26.4%, CBD<1%), Bedrolite (THC<1%, CBD=6.0-9.0%) or Bediol (THC=5.0-7.6%, CBD=6.4-9.6%) [5]. Nonetheless, in recent years the scientific community has focused its attention to the *Cannabis* phyto-complex as a whole. In fact, the beneficial properties of this plant can also be ascribed to or modulated by other components of the plant, such as the terpenes, producing a synergistic effect also known as *entourage effect* [6–10].

The administration of *Cannabis,* nowadays, can follow many routes, but it seems that *Cannabis* oils administered through the sublingual or buccal surface can produce higher bioavailability of the active compounds, while guaranteeing more adjustable dosages and acceptably rapid onset [11]. Of course, the bioactive components have to be decarboxylated prior to the preparation of the final pharmaceutical form, and for this purpose the Italian jurisdiction suggests the setting up of the *Cannabis* oils according to prescription, DAB Pharmacopoeia or

different methods found in scientific literature, such as those promoted by Romano and Hazekamp, Citti and Cannazza, Società Italiana Farmacisti Preperatori (SIFAP), Calvi and Pacifici [12–16].

The first two methods require *Cannabis* to be mixed with olive oil or ethanol. The attained mixture is then heated (for two hours in a boiling water bath according to the Romano-Hazekamp method or for two hours in olive oil at 110 °C according to the Citti-Cannazza method) and then filtered to obtain the oily extract [12,13]. The SIFAP method, on the other hand, requires to initially cut the *Cannabis* inflorescences and to preheat them at 115 °C for 40 minutes in an oven; the drug is preheated by spreading it in a layer 1 cm high. *Cannabis* is then transferred into the olive oil and further crushed with a turbo emulsifier for 3 minutes. The mixture of *Cannabis* and olive oil is then heated in a boiling water bath for 40 minutes, filtered and added with 0.05% w/v alpha-tocopherol as an antioxidant [14].

The method promoted by Pacifici involves preheating the plant material at 145 °C for 30 minutes and subsequently heating it in a boiling water bath for two hours [16]. Ultimately, the Calvi method involves a heating step of the plant material prior to the extraction step which is then performed using an ultrasonic water bath [15]. All the methods above described require the weight/volume ratio (g/mL) of drug and solvent to be 0.1 g/mL.

According to our expertise in producing *Cannabis* oils, the current processes for the extraction of *Cannabis,* with the purpose of obtaining a cannabinoids enriched oil, do not allow a significant extraction of neither THC nor CBD while preserving chemical stability over time. Therefore, the aim of our work was to evaluate the stability of both cannabinoids and terpenes in three different varieties of *Cannabis* (Bedrocan, Bedrolite and Bediol) over eight months of the oils prepared with the hereby proposed method of cannabinoids extraction.

Results

The aim of our work was to assess the stability of *Cannabis* oils using a new extraction method, three different *Cannabis* varieties and olive oil as carrier. Stability was assessed over a period of eight months once a month. Each sample was stored in a closed container and at different temperatures (25 °C and 5 °C). Half of the samples were added with tocopherol acetate (0.05% w/v), as described in Materials and Methods. Before each analysis, the samples were shaken, simulating what the patient is recommended to do before use. Once the amount for analysis was gathered, each sample was closed and placed in the appropriate temperature conditions.

The three used variety of *Cannabis* have different concentrations of both THC and CBD; particularly, Bedrocan contains meanly 22% of THC, Bediol 6.3% of THC and 8.0% of CBD, and Bedrolite 7.5% of CBD. Of course, these distinctions were reflected on the concentrations of active principles of the oils, whereas Bedrocan preparations had an initial concentration of THC = 1.53±0.01 % (w/w), Bediol of THC = 0.30±0.006 % (w/w) and of CBD = 0.43±0.01 % (w/w), and Bedrolite of CBD = 0.67±0.005 % (w/w), as displayed in Fig. 1. As far as it regards the stability of these active principles, no peculiar differences were noted between samples with and without antioxidant and at different storage temperatures until 8 months, as the THC and CBD concentrations were within a ±10% range. Unexpectedly, after the whole study period, THC concentrations seem to decrease in refrigerated samples. All other samples appear to be stable over the whole study period (Fig. 1).

The terpene profiles of the three varieties in study was assessed at the beginning of the study, as shown in Fig. 2. To evaluate the stability of the terpenes over time, only those that were present in adequate and robust quantities in all the samples of the different varieties, namely β-Caryophyllene and α-Humulene, were taken into consideration, monitoring their concentration over a period of eight months. In Bedrocan oils, at room temperature both the analytes remain stable over time, while at 5 °C they begin to drop from within five months (β-Caryophyllene -30%, and α-

Humulene -50%, Fig. 3). Moving on to Bediol (Fig. 4) both β-Caryophyllene and α-Humulene seem to decrease over time at both temperatures, although at 25 °C this phenomenon is less noticeable, as α-Humulene is completely undetectable in refrigerated samples after four months only. Finally, in Bedrolite preparations a trend similar to the one observed in Bedrocan can be appraised; nevertheless, the concentrations of both analytes seem to decrease in a shorter time, within four months, in a quite greater extent (β-Caryophyllene -50%, and α-Humulene -50%, Fig. 5). In all cases, no differences were noted between samples with and without antioxidant.

Foremost, it was pivotal to evaluate more parameters other than cannabinoids and terpenes concentrations and stability. For example, the residual ethanol content in the final product had to be assessed; using head-space gas chromatography (see supporting information file for method elucidation), we found that the ethanol content was far below the limits imposed by the European Pharmacopoeia (<0.05% w/w). Besides, the ethanol distilled from the extract could be re-used up to 25 times without showing any contamination from cannabinoids nor terpenes (data not shown). Finally, we decided to measure the residual content of water, using the Karl-Fisher method, and it was under 0.05%; the analysis was performed using an automatic Karl-Fisher analyser (V20 Volumetric KF Titrator, Mettler-Toledo S.p.A., Milan, Italy) [17].

Discussion

The concentrations of cannabinoids, namely THC and CBD, resulted to be stable along the whole period of the study in all three varieties of *Cannabis.* As appraisable, though, in the long-term THC did not seem to be particularly stable at refrigerated temperatures; foremost it is quite safe to state that the stability of the extracts is not remarkably influenced by the presence of antioxidants, as already proved in previous experiments [9].

Likewise, terpenes and terpenoids, according to the monitored concentrations of β-Caryophyllene and α-Humulene, appear to be more consistent when the oils are store at room temperature and, seemingly to cannabinoids, they are not affected by the addition of antioxidants. It is worth noticing, though, that despite the increasing evidence of the contribution of the *entourage effect* of *Cannabis* phytocomplex [6–8], there is still no legal requirements for the concentrations of terpenes in *Cannabis* oleolites.

Compared to previously used methods, the method hereby presented gives back comparable yield in terms of cannabinoids content (>85%) and different oils carriers can be used according to the patients' preferences/taste and desired bioavailability, while guaranteeing more safety to the operator thanks to the room temperature extraction, which only happens using the Calvi extraction method [15]. Nonetheless, this method has the opportunity to offer many advantages, especially to the technological side of the preparation. In fact, on one hand, the stability of the product at room temperature can help avoiding solidification issues of the refrigerated conditions and resulting dosage biases due to inhomogeneous material; on the other, the preparation of an extremely highly concentrated matrix brings to a vast flexibility in controlling the concentration of the extract in order to produce highly concentrated oils and rises the possibility of producing standardized oils despite the variability of the starting plant material; in fact, this way it is possible to know the concentrations of active principles prior to the dilution in oil by simply analysing the extracted material. While the previously described methods only rely on the extraction driving force of the oily matrix [12–16] and each preparation requires a unique analytical evaluation, with this procedure it is possible to prepare a high amount of decarboxylated cannabinoids to be distributed in different formulations, that might require different cannabinoids concentrations. Preliminary experiments may let us speculate that the clear separation of the extraction and decarboxylation steps allows a precise setting and control of the share of acid (mainly THCA, CBDA) and neutral forms (mainly THC and CBD) in hybrid formulations containing both acid and neutral forms in

different percentages; foremost, terpenes preservation may be improved due to the decarboxylation step taking place after extraction and not on the starting plant material as in all other extraction methods [12–16]. Eventually, preliminary studies suggest that the active principles could be more easily absorbed because of the higher particle surface of the extracted matrix and the highly concentrated residue may be used as the basis for further processing such as emulsification and spray-drying to obtain other pharmaceutical forms namely Dry Powders Inhalation (DPI), sublingual and buccal oromucosal powders, though further technological studies are necessary. This preparation method is currently in use in the compounding laboratory of Farmacia Caputo and it is the method of choice when not explicated by the prescriber. To this day, the pharmacy is capable of producing up to two thousand oils per year.

As these premises were given, further and deeper studies are surely necessary for a more substantial comprehension of these extracts, nonetheless the hereby presented procedure could be a useful starting point for an innovation in cannabinoids, terpenes and terpenoids extraction.

Materials and Methods

Chemicals and reagents

The terpenes standards, analytical grade solvents and 4-Vinyl-1-cyclohexene (used as internal standards) were purchased from Sigma-Aldrich. d5-Linalool (used as internal standard) was purchased from HPC Standards GmbH. Bedrocan, brand name for the cultivar Cannabis sativa L. 'Afina', produced by BEDROCAN BV (Veendam, Nederland) is available in dried whole flower. It is declared to have a balanced ratio of THC 17,6%–26,4% and CBD <1%. Bediol, brand name for the cultivar Cannabis sativa L. 'Elida', produced by BEDROCAN BV (Veendam, Nederland) is available in dried granulate flower. It is declared to have a balanced ratio of THC 5,0%–7,6% and CBD 6,4%-9,6%. Bedrolite brand name for the cultivar Cannabis sativa L. 'Rensina', produced by

BEDROCAN BV (Veendam, Nederland) is available in dried granulate flower. It is declared to have a balanced ratio of THC <1% and CBD 6,0%-9,0%. The voucher specimens consisted of dried female inflorescences. The voucher specimen for the marketed C. sativa was preserved and deposited at the Ghirardi Botanical Garden of the Department of Pharmaceutical Sciences of the University of Milan under the accession number 021/DISFARM, as stated in [14].

Refined olive oil Ph. Eur. was obtained from J.H.Mueller (Hanstedt, Germany). Fatty acid composition of used batch was 1.30% palmitoleic (C16:1), 12.20% palmitic acid (C-16:0), 1.30% palmitoleic acid (C-16:1), 3.50% stearic acid (C-18:0), 74.70% oleic acid (C-18:1), 6.70% linoleic acid (C-18:2), 0.80% linolenic acid (C-18:3), 0.40% arachic acid (C-20:0), 0.20% eicosenoic acid (C-20:1), 0.10% behenic acid (C-22:0), and <0.10% lignoceric acid (C-24:0). Pharmaceutical grade ethanol (96% V/V) was obtained from Vital srl, (Milan, Italy).

Oleolites samples

The oleolites were made using three different varieties of *Cannabis*, namely Bedrocan, Bediol and Bedrolite. For each variety, n. 4 samples were prepared, two of which were added with tocopherol acetate (0.05% w/v) as an antioxidant, for a total amount of n. 12 samples. The stability of cannabinoids and terpenoids was assessed over eight months, once a month. Samples were stored sealed at both room temperature (25±2°C) and refrigerated temperature (5±3° C), so that for each variety the stability of the samples with and without antioxidant was assessed at two different temperatures. Samples are identified as A (with antioxidant, stored at room temperature), B (without antioxidant, stored at room temperature), C (with antioxidant, stored at refrigerated temperature), D (without antioxidant, stored at refrigerated temperature). Prior to the analysis, the samples were thoroughly mixed, in order to simulate the shaking of the product the patient would do before self-administration.

Cannabis extraction method

An exact amount of *Cannabis* inflorescences (20 g), according to the patient's prescription, is weighed, and put in a grinding machine (Tube Mill 100 control, Ika, Staufen im Breisgau, Germany). The grinding chamber is frozen and the inflorescences ground for 15 seconds at 10.000 rpm. The resulting mince is dispersed in 400 mL of cold ethanol Ph.Eu. 96% V/V and an additional grinding is performed, in order to homogenize the matrix, with a turbo-emulsifier (Silverson L5M-A, Crami Group srl, Italia). for 60 seconds at 5.000 rpm. After that, the resulting mixture is sonicated using ultrasonic waves under stirring; the extraction is conducted using an ultrasonic system equipped with a S24d14D sonotrode with a 100% amplitude (UP400St, 400W, 24kHz, Hielscher, Teltow, Germany); during the sonication/extraction process the temperature of the mixture of plant material and ethanol is maintained below 25° C by using an ice bath in order to perform a most efficient as possible extraction and minimize the loss of volatile components. The sonication/extraction step is performed for a time ranging from 10 to 15 minutes depending on the variety of flower (Table 1). After that, the mixture is vacuum filtered, and the filtrate evaporated under reduced pressure at a temperature of 40 °C, using a rotary evaporator (IKA RV 10 digital DS 99, Staufen im Breisgau, Germany). Finally, the highly concentrated residue is decarboxylated in a static oven (Memmert UN55 plus, Memmert, Büchenbach, Germany) at a temperature of 130° C for a time ranging from 30 to 60 minutes depending on the variety of the material (Table 1). At last, the dried extract, accurately weighed, is resuspended in an appropriate amount of oil (olive oil, as in this case, MCT, other carrier oils) to reach the desired concentration, according to prescription.

For the purpose of this work, only olive oil preparations were produced and the extraction procedure parameters are displayed in the following table (Table 1).

Determination of cannabinoids

Sample preparation

50 mg of the *Cannabis* oil were weighed using an analytical scale (QUINTIX224-1S, Sartorius) and diluted with 5 mL of methanol, vortexed and centrifuged (1789 xg, 5 min). 900 µL of supernatant were withdrawn in a glass vial and 5 µL injected in the HPLC/UV system.

HPLC/UV analysis

The HPLC/UV system in use is a HPLC/UV Prominence-i LC-2030C-Cannabis Analyzer for Potency (Shimadzu Corporation). The column in use was a reversed-phase Shimadzu NexLeaf CBX for Potency, 2.7 µm (150 mm x 4.6 mm), equipped with a security guard cartridge. Chromatographic separation was achieved using a linear gradient between eluent A (water) and eluent B (acetonitrile) both containing 0.085 % phosphoric acid. The flow rate was 1.6 mL/min and the column temperature was 35 °C. The elution gradient was set as below: 0 -7 min (70–85 % B), 7.0–7.1 min (85–95 % B), 7.1–8.0 min (95 % B), 8.0–8.1 min (95-70 % B) and 8.1 -10 min (70 % B). The UV detection was monitored at a fixed wavelength of 220 nm [18]. The validation of the method on this matrix can be found in the supporting information file (Tables 1S-3S)

Determination of terpenes and terpenoids

Sample preparation

100 mg of the *Cannabis* oil were weighed, withdrawn in a 20 mL headspace vial with 10 µL of IS solution (4-vinylcyclohexene and d5-linalool 2 mg/mL) and 10 µL of methanol. The vial was then processed as below described.

SHS-GC/MS-Q analysis

GC–MS analyses were carried out on a 6890 Series Plus gas chromatograph equipped with an Agilent 7683 static headspace autosampler and coupled to a 5973N mass selective detector (Agilent Technologies). Terpenes and terpenoids were analysed using a static headspace sampling (SHS) method, whose parameters were: oven: 160 °C; manifold: 180 °C; transfer line: 200 °C; incubation time: 15 min; interval: 45 min; N_2 overpressure: 0.5 psi. The chromatographic separation was achieved with a DB-5MSUI capillary column (5%diphenyl/95%dimethylpolysiloxane, 0,18μm, 20 m x 0.18mm, Agilent Technologies). The GC/MS conditions were: injector temperature: 250 °C; injection flow: 43.9 mL/min; split flow: 39.8 mL/min; split ratio: 80:1; oven temperature program: initial 40 °C, 5 °C/min up to 200 °C; solvent delay: 1 min. The MS detector was operated in full scan mode, acquiring ions from m/z 40–300, with a rate of 5 spectra/s, ion source was set to 230 °C and the transfer line to 280 °C. The total analysis time was 32 min. Identification of terpenes was performed by (1) comparison with a series of standard solutions consisting of 35 terpenoids and/or (2) matching mass spectra with NIST library (ver. 2017). The quantitative data were determined by comparing the extracted base peak areas for each analyte, corrected for internal standards responses, against a calibration curve. The validation data for β-Caryophyllene and α-Humulene are reported in supporting information file (Tables 4S-6S).

Data visualization

Graphs were prepared with GraphPad Prism 7.0 (GraphPad Software, Inc).

Supporting Information

Ethanol head-space gas chromatography method; validation parameters for the methods concerning cannabinoids and terpenes/terpenoids analyses.

Conflicts of Interest

The authors declare no conflicts of interest.

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Figures

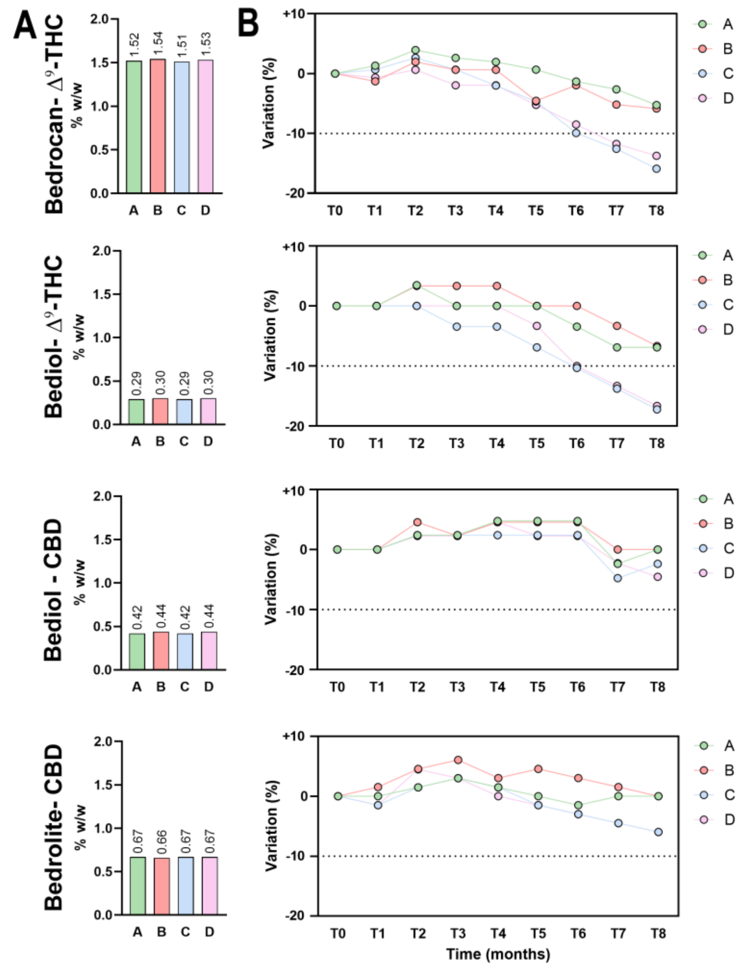

Fig. 1. Panel A displays the concentrations (% w/w) of cannabinoids in each sample at the beginning of the study (T0); panel B displays the variation (%) of cannabinoids concentrations over time. Samples A were added with tocopherol acetate (0.05% w/v) as an antioxidant and stored at room temperature. Samples B were not added with an antioxidant and stored at room temperature. Samples C were added with tocopherol acetate (0.05% w/v) as an antioxidant and stored at refrigerated temperature. Samples D were not added with an antioxidant and stored at refrigerated temperature. The number after "T" refers to the months passed after the beginning of the study; therefore, T0 indicates the beginning of the study, T1 one months later and so on.

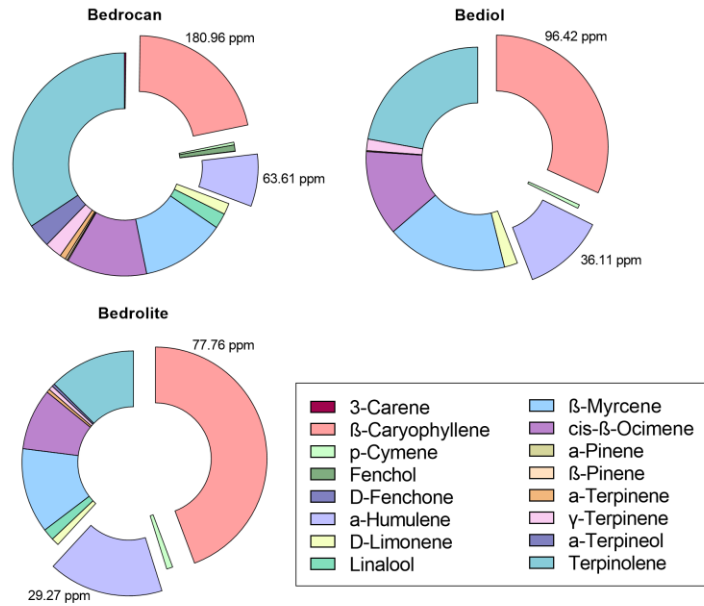

Fig. 2. Initial composition of the terpenes and terpenoids profile in all three *Cannabis* varieties. Exploded sections of the graphs represent β-Caryophyllene (pink) and α-Humulene (lilac), whose concentrations (ppm) are explicit. For abbreviations, see legend of Fig. 1.

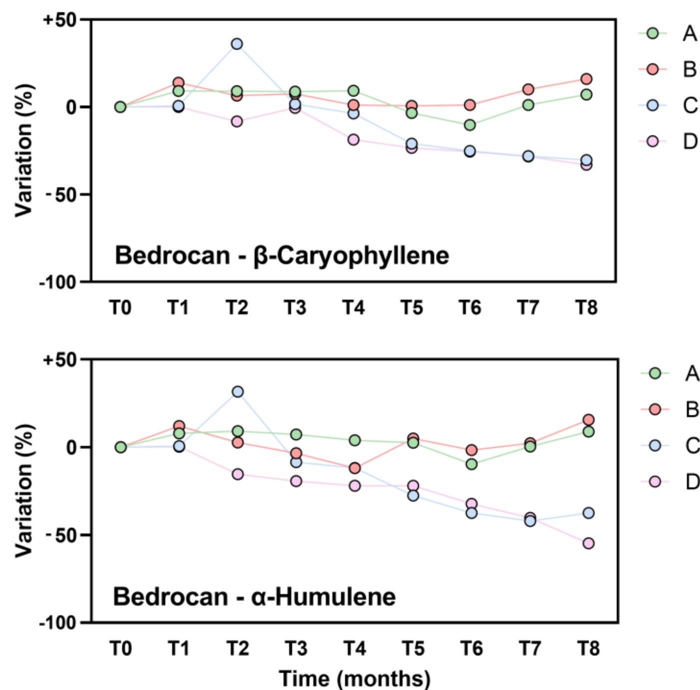

Fig. 3. Variation (%) of the concentrations of β-Caryophyllene and α-Humulene in Bedrocan samples over time. For abbreviations, see legend of Fig. 1.

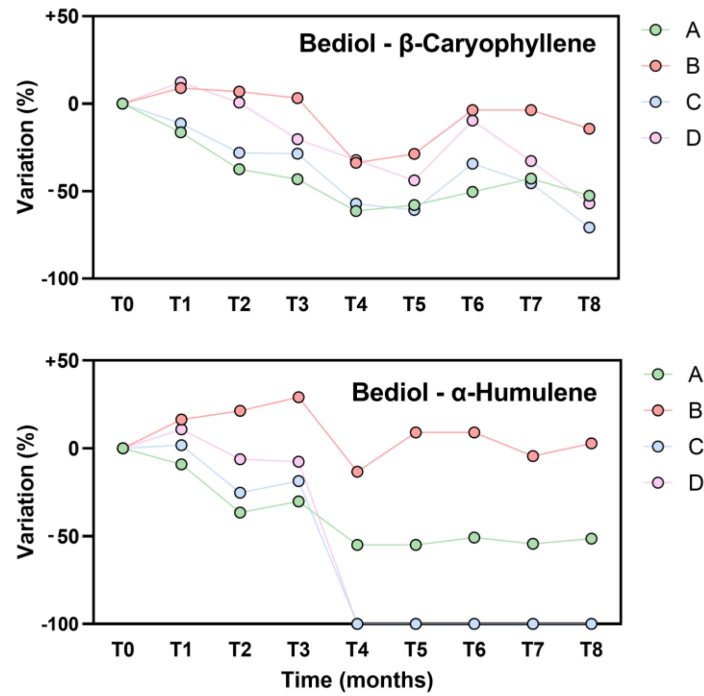

Fig. 4. Variation (%) of the concentrations of β-Caryophyllene and α-Humulene in Bediol samples over time. For abbreviations, see legend of Fig. 1.

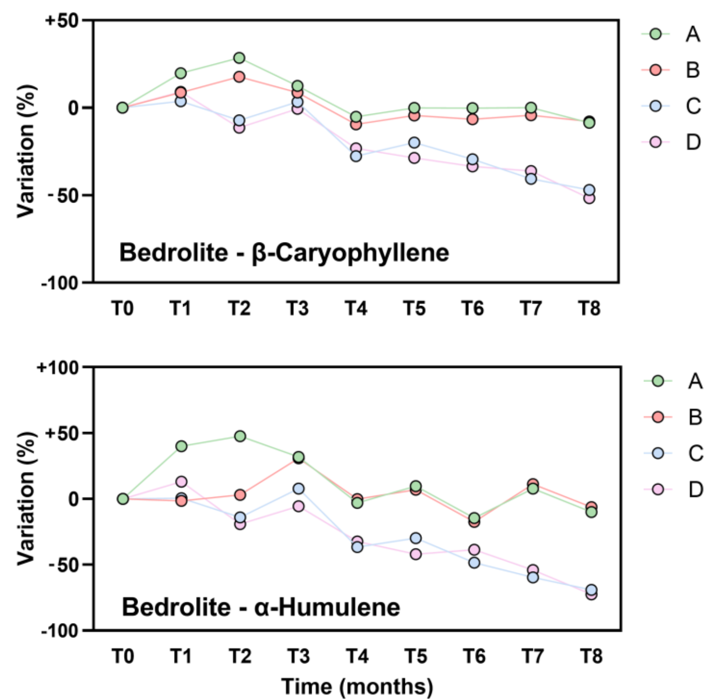

Fig. 5. Variation (%) of the concentrations of β-Caryophyllene and α-Humulene in Bedrolite samples over time. For abbreviations, see legend of Fig. 1.

Tables

Table 1. Cannabinoids extraction procedure for the different varieties in use in this study.

Variety	Starting Material (g)	Extraction time (min)	Weight of the extract (g)	Decarboxylation time (min)	Weight after decarboxylation (g)
Bedrocan	20	10	6.24	30	5.37
Bediol	20	13	4.49	40	3.90
Bedrolite	20	15	3.24	60	2.76

Supporting Information

Medicinal *Cannabis*: extended stability of *Cannabis* extracts produced using a new ethanol-based extraction method

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Ethanol head-space gas chromatography method

HS-GC-FID analyses for ethanol impuritites were carried out on a TraceGC gas chromatograph equipped with a HS2000 headspace autosampler and coupled to a FID detector (Thermo Electron). Autosampler parameters were: oven: 100 °C; incubation time: 30 min. The chromatographic separation was achieved with a VF-624MS capillary column (6%Cyanopropyl-phenyl-94%methylpolysiloxane, 1.8μm, 30 m x 0.32mm, Agilent Technologies). The GC-FID conditions were: injector temperature: 200 °C; split ratio: 80:1; oven temperature program: initial 50 °C, 10 °C/min up to 200 °C; detector temperature: 280 °C. Ethanol retention time was 2.41 min; n-propanol (used as internal standard) retention time was 3.32 min.

Table 1S Analytes concentrations for QC and calibration ranges for CBDA, CBD, THCA and THC.

Analytes	Concentrations (% w/w)	Calibration ranges (% w/w)
CBDA, CBD, THCA and THC	0.05	0.05-1
	0.1	
	0.2	
	0.4	
	0.5	
	1	

Table 2S Calibration parameters for CBDA, CBD, THCA and THC.

Analyte	r^2	LOD (% w/w)	LOQ (% w/w)
CBDA	0.999	0.0025	0.005
CBD	0.999	0.0025	0.005
THCA	0.998	0.0025	0.005
THC	0.999	0.0025	0.005

Table 3S Precision and accuracy for CBDA, CBD, THCA and THC.

	Precision		Accuracy	
Analyte	**Amount (% w/w)**	**CV%**	**Amount (% w/w)**	**Er%**
CBDA	0.5	2.10	0.5	1.47
CBD	0.5	1.72	0.5	1.54
THCA	0.5	2.50	0.5	4.35
THC	0.5	2.21	0.5	5.26

Table 4S Analytes concentrations for QC and calibration ranges for β-Caryophyllene and α-Humulene.

Analyte	Concentrations (ppm)	Calibration ranges (ppm)
β-Caryophyllene	15	15-200
	25	
	50	
	100	
	150	
	200	
α-Humulene	5	5-100
	12.5	
	25	
	50	
	75	
	100	

Table 5S Calibration parameters for β-Caryophyllene and α-Humulene.

Analyte	r^2	LOD (ppm)	LOQ (ppm)
β-Caryophyllene	0.998	1.844	10
α-Humulene	0.997	0.073	2.5

Table 6S Precision and accuracy for β-Caryophyllene and α-Humulene.

	Precision		Accuracy	
Analyte	**Amount (ppm)**	**CV%**	**Amount (ppm)**	**Er%**
β-Caryophyllene	20	8.18	15	4.76
	40	0.91	45	4.37
	60	1.95	65	4.06
α-Humulene	10	3.17	7.5	1.22
	20	0.84	22.5	2.75
	30	2.92	32.5	2.89