Medicinal cannabis: Principal cannabinoids concentration and their stability evaluated by a high performance liquid chromatography coupled to diode array and quadrupole time of flight mass spectrometry method

Cinzia Citti [a,b], Giuseppe Ciccarella [a,b], Daniela Braghiroli [c], Carlo Parenti [c], Maria Angela Vandelli [c], Giuseppe Cannazza [b,c,*]

[a] Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Universitaèl Salento, Via per Monteroni, 73100 Lecce, Italy
[b] CNR NANOTEC, Campus Ecoteckne dell'Università del Salento, Via per Monteroni, 73100 Lecce, Italy
[c] Dipartimento di Scienze della Vita, Università di Modena e Reggio Emilia, Via Campi 103, 41125 Modena, Italy

ARTICLE INFO

ABSTRACT

In the last few years, there has been a boost in the use of cannabis-based extracts for medicinal purposes, although their preparation procedure has not been standardized but rather decided by the individual pharmacists. The present work describes the development of a simple and rapid high performance liquid chromatography method with UV detection (HPLC-UV) for the qualitative and quantitative determination of the principal cannabinoids (CBD-A, CBD, CBN, THC and THC-A) that could be applied to all cannabis-based medicinal extracts (CMEs) and easily performed by a pharmacist. In order to evaluate the identity and purity of the analytes, a high-resolution mass spectrometry (HPLC-ESI-QTOF) analysis was also carried out. Full method validation has been performed in terms of specificity, selectivity, linearity, recovery, dilution integrity and thermal stability. Moreover, the influence of the solvent (ethyl alcohol and olive oil) was evaluated on cannabinoids degradation rate. An alternative extraction method has then been proposed in order to preserve cannabis monoterpene component in final CMEs.

1. Introduction

In the last few years, the use of cannabis-based extracts for therapeutical purposes has greatly increased. Nonetheless, cannabis is out of tune with the conventional paradigm of design, development and testing which generally applies to medicines. Indeed, it was pioneered and promoted by patients and their caregivers, instead of scientific researchers or physicians. It is often consumed in its herbal form, using unconventional modes of intake such as smoking, vaporizing, tea or brownies [1]. Moreover, despite the release onto the market of the main cannabis active principle, (−)-Δ^9-*trans*-tetrahydrocannabinol ((6aR,10aR)-delta-9-tetrahydrocannabinol or Δ^9-THC, Fig. 1), as a synthetic derivative, the employment of the drug or its medicinal extracts is still widespread. This is due to the higher efficacy towards the treatment of specific pathologies and to a lower onset of side effects compared to the synthetic drug [2,3]. In fact, cannabis-based medicinal extracts (CMEs) can improve neurogenic symptoms unresponsive to standard treatments [4]. Even though still very little is known about the chemical composition of CMEs, more and more in-depth studies of the pharmacological properties of the different active principles present in cannabis inflorescences have allowed for their application in several pathologies [5,6]. Indeed, cannabis is used to reduce nausea and vomiting during chemotherapy, to improve appetite in people with HIV/AIDS, to treat chronic pain and help with muscle spasms [7,8].

Since the chemical composition largely varies among the cannabis varieties employed the Bedrocan BV company has recently developed a few cannabis strains with a standardized composition of cannabinoid active principles. In particular, the
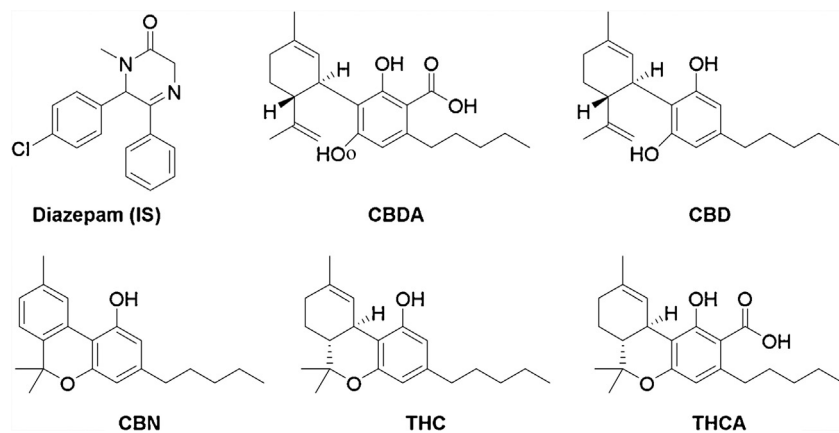
Fig. 1. Molecular structure of Diazepam (IS), CBD-A, CBD, CBN, THC and THC-A.

Bedrocan® strain contains 22% (w/w) THC, while the Bediol® strain contains 6.3% (w/w) THC and 8% (w/w) cannabidiol (CBD, Fig. 1). Other strains are Bedrobinol® with 13.5% (w/w) THC, Bedrolite® with <0.4% (w/w) THC and 9% (w/w) CBD and Bedica® with 14% (w/w) THC. The main cannabinoids THC and CBD are found in the plant in the carboxylated form (tetrahydrocannabinolic acid or THC-A and cannabidiolic acid or CBD-A, Fig. 1). Although these compounds possess several pharmacological properties, they do not display psychotropic activity simply because they are not able to cross the blood-brain barrier. When subjected to heat they undergo decarboxylation to give the psychoactive THC and CBD. THC is the main responsible for the psychotropic activity known for cannabis, whilst CBD seems to have analgesic and antioxidant activity and to reduce THC side effects [9–11]. If cannabis is exposed to air for a prolonged period of time, THC-A will convert to cannabinolic acid (CBN-A). Decarboxylation of CBN-A will then give cannabinol (CBN). CBN (Fig. 1) is a weak psychoactive cannabinoid with mostly anticonvulsant activity [12] and mild analgesic properties [13].

Anyway, it has been widely demonstrated that the effect of the single cannabinoids is different from that of the whole CME [14,15]. It has been suggested that cannabinoids in their acidic form present in CMEs can also intervene in the pharmacological activity [16]. Hence, it is important to determine the main cannabinoids in both their forms in order to correlate the individual amount present in the CMEs with the pharmacological effects. To this end, the most widely employed methodology is gas chromatography [17,18]. Anyway, this method involves the heat of the mixture prior to its chromatographic separation resulting in the partial decarboxylation of the cannabinoids in their acid form. Therefore, this methodology cannot be employed to evaluate the actual composition of cannabinoids present in the cannabis-based preparations without a preliminary derivatization step. Liquid chromatography (LC) based methods, instead, do not cause any sample decomposition since the analysis is conducted at room temperature. The literature reports various LC-based methods with either ultraviolet (UV) or mass spectrometry (MS) detection for the qualitative and quantitative determination of cannabinoids [18–22]. Although liquid chromatography enables the determination of the cannabinoid composition, it does not allow to establish the terpenoid composition of CMEs. In this case, the use of gas chromatography is mandatory.

Even though there is a large increase in the use of cannabis-based preparations for medicinal purposes [23], their preparation procedure has not been standardized but rather decided by the individual pharmacists. Recently, the Italian legislation has imposed the qualitative and quantitative determination of the main cannabinoids in each CME by a specific and selective chromatographic method [24]. The primary goal of this work was to develop a simple and cost effective chromatographic method that can be easily performed by a pharmacist who dispenses his own CMEs. The method developed has allowed to evaluate how the cannabinoids composition (acid and neutral form) can change depending on the temperature and time of extraction of the drug. In order to evaluate the identity and purity of the analytes, a high-resolution mass spectrometry analysis was also carried out.

It has been recently suggested that the volatile terpenoid composition can also contribute to the pharmacological activity of cannabis-based drugs [2,25]. Therefore, it is important to avoid any loss in terpenes, which is caused by their evaporation. Hence, a simple way to preserve such component in CMEs has been proposed. Gas chromatography coupled to mass spectrometry (CG-MS) analyses were performed in order to evaluate the variation in the terpenoid composition during the preparation.

2. Material and methods

2.1. Materials

All chemicals and reagents, except those specifically noted, were purchased from Sigma-Aldrich. Acetonitrile, water, 2-propanol, formic acid LC–MS grade was purchased from Carlo Erba (Milan, Italy). Special refined olive oil was bought from Fagron Italia Srl (Bologna, Italy). Ethyl alcohol was of pharmaceutical grade bought from Carlo Erba (Milan, Italy). THC and CBD were purchased from SALARS (Como, Italy). CBN, THC-A and CBD-A from Echo Pharmaceuticals B.V. (Weesp, The Netherlands). All CMEs (Bediol® olive oil, ethyl alcohol and carbon dioxide medicinal extracts) were prepared by Farmacia Tundo Dr. Alfredo (Alliste, Lecce, Italy) employing a final ratio of 1 g of cannabis inflorescence in 10 mL of either olive oil or ethyl alcohol.

2.2. LC-UV analysis

High performance liquid chromatography (HPLC) analyses were performed on an Agilent Technologies (Waldbronn, Germany) modular model 1200 system, consisting of a vacuum degasser, a binary pump, a thermostated autosampler, a thermostated column compartment and a diode array detector (DAD). The chromatograms were recorded using an Agilent Mass Hunter software (Rev. B.01.04). A Poroshell 120 C18 column (Poroshell 120 SB-C18, 2.1 × 100 mm, 2.7 μm, Agilent, Milano, Italy) was used with a mobile phase composed of 0.1% formic acid in both (A) water and (B) acetonitrile (ACN). The isocratic elution was set at a flow rate of 0.5 mL/min. The run time was 10 min. The column temper-

ature was set at 25 °C. The sample injection volume was 5 μL. The UV/DAD acquisitions were carried out in the range 190–500 nm and chromatograms were acquired at 228 nm. Three injections were performed for each sample.

2.3. LC–MS analysis

LC–MS and MS² analyses were performed using an Agilent Technologies modular 1200 system, equipped with a vacuum degasser, a binary pump, a thermostated autosampler, a thermostated column compartment and a 6540 quadrupole time of flight (QTOF) mass analyzer with an electrospray ionization (ESI) source. The HPLC column and the applied chromatographic conditions were the same as reported for the HPLC-UV/DAD system. The HPLC-ESI–MS system was operated in positive ion mode. The experimental parameters were set as follows: the capillary voltage was 3.5 kV, the nebulizer (N_2) pressure was 35 psi, the drying gas temperature was 350 °C, the drying gas flow was 11 L/min and the skimmer voltage was 40 V. Data were acquired by Agilent Mass Hunter system software (version 6.0). The mass spectrometer was operated in full-scan mode in the m/z range 50–500. MS² spectra were automatically performed with nitrogen as the collision gas in the m/z range 50–1700, using the auto MS/MS function and a collision energy of 20 eV. Extracted ion chromatograms (EICs) were obtained with an accuracy of 10 ppm m/z from total ion chromatogram (TIC) employing the m/z corresponding to [M+H]$^+$ 285.0770 for diazepam (Fig. 1) as internal standard (IS), 359.2224 for CBD-A, 315.2314 for CBD, 311.2000 for CBN, 315.2311 for THC and 359.2216 for THC-A. Peak areas were calculated employing the EICs.

2.4. GC–MS analysis

Chromatographic analyses were carried out on a GC 6890N (Agilent Technologies, Waldbronn, Germany), coupled to a 5973 Network mass spectrometer (Agilent Technologies). Compounds were separated using a HP-5 MS cross-linked 5% diphenyl-95% dimethyl polysiloxane capillary column (30 m × 0.25 mm I.D., 1.00 μm film thickness, Agilent Technologies). The oven temperature was programmed from initial 40 °C to final 280 °C at 3 °C/min, which was maintained for 5 min. The injection volume was 1 μL, with a 1:100 split ratio. Helium was used as the carrier gas at a flow rate of 0.7 mL/min. The injector, transfer line and ion source temperatures were 230, 280 and 230 °C, respectively. MS detection was performed with electron ionization (EI) at 70 eV, operating in full-scan acquisition mode in the m/z range 40–400. Monoterpenes α-Pinene, β-pinene, myrcene, limonene, cis-ocimene, terpinolene and sesquiterpenes β-cariophyllene, α-guaiene and humulene were identified by comparing the retention time of the chromatographic peak with that of authentic reference compounds run under the same conditions. The comparison of the MS fragmentation pattern of the target analytes with that of pure compounds was performed. Mass spectrum database search was carried out using the National Institute of Standards and Technology (NIST) mass spectral database (version 2.0d, 2005).

2.5. Preparation of standard solutions

Blank matrices for olive oil and for ethyl alcohol CMEs were obtained by mixing, respectively, 50 μL of olive oil or 50 μL of ethyl alcohol with 9950 μL of 2-propanol. Stock solution of CBD-A, CBD, CBN, THC and THC-A (1000 μg/mL) in methanol or ethanol were properly diluted in the blank matrices to obtain samples of 1.00, 5.00, 10.0, 50.0 and 100 μg/mL. 100 μL of each samples were diluted in 890 μL of mobile phase and 10 μL of IS (500 μg/mL) to the final concentrations of 0.10, 0.50, 1.00, 5.00 and 10.0 μg/mL for CBD-A, CBD, CBN, THC and THC-A and 5 μg/mL for IS. Indepen-

dently prepared CBD-A, CBD, CBN, THC and THC-A stock solutions of 1.00, 2.50, 25.0 and 75.0 μg/mL were used for the preparation of lower limit of quantification LLOQ (0.10 μg/mL), low concentration quality control (LQC) (0.25 μg/mL), medium concentration quality control (MQC) (2.50 μg/mL), and high concentration quality control (HQC) (7.50 μg/mL) samples, respectively. QC samples were prepared as for calibration standards. A stock solution of IS (5000 μg/mL) was prepared by dissolving 50 mg in 10 mL of acetonitrile. Working solution was prepared by further diluting the stock solution in mobile phase to 500 μg/mL.

2.6. Method validation

In order to demonstrate the reliability of the method, full method validation was carried out based on EMEA guidelines [26] and in agreement with international guidelines for analytical techniques for the quality control of pharmaceuticals (ICH guidelines) [27]. The validation was performed for both LC-UV and LC–MS methods. The fully validated method has been transferred to two Italian pharmacies, Farmacia Dr. Ternelli (Bibbiano, Reggio Emilia, Italy) and Farmacia Tundo Dr. Alfredo, that currently apply it to analyze CMEs.

2.6.1. Specificity and selectivity
The specificity of the assay was investigated by analysis of blank samples and the retention times of the interfering compounds in olive oil or ethyl alcohol were compared with those of authentic standards and IS. The detector employed was a UV/DAD; the spectra acquired during the elution of each peak corresponding to each cannabinoid are normalized and overlaid for graphical presentation. Moreover, identification was assessed by comparing accurate (within 2 ppm error) m/z of [M+H]$^+$ and MS/MS spectra of authentic standards with those obtained by LC-ESI-HRMS and MS/MS of samples.

2.6.2. Linearity
Calibration curve was constructed at five calibration levels 0.50, 2.50, 5.00, 25.0 and 50.0 ng of CBD-A, CBD, CBN, THC and THC-A and 2.50 ng of diazepam (IS). Peak area ratios of drug-to-IS were plotted vs. actual concentrations. Calibration curve was evaluated at the beginning of each validation day of five consecutive days ($n = 5$). Linearity was assessed through evaluation of the coefficient of determination (R^2), which should be greater than 0.998 using weighted regression method (1/x).

2.6.3. Limit of detection (LOD) and limit of quantification (LOQ)
Limit of detection (LOD) was estimated based on a 3:1 signal-to-noise ratio. Standard stock solutions of all analytes were appropriately diluted at the levels of these estimated values and were analyzed repeatedly six times. Limit of quantification (LOQ) was estimated based on a 10:1 signal-to-noise ratio. Standard stock solutions of all analytes were appropriately diluted at the levels of these estimated values and were analyzed repeatedly six times.

2.6.4. Precision and accuracy
The precision and accuracy were evaluated at four levels, LLOQ (0.50 ng), LQC (1.25 ng), MQC (12.5 ng), and HQC (37.5 ng). Each sample was analyzed repeatedly three times within a single day to determine the intra-day precision and accuracy, and three times a day for five successive days ($n = 15$) to determine the inter-day precision and accuracy. The precision was expressed as the coefficient of variance (RSD), and the accuracy was expressed as the percentage of mean calculated to nominal concentration.

2.6.5. Recovery

Recovery was evaluated using olive oil and ethyl alcohol CMEs spiked at three different levels with standards. To this end, a standard solution was added to the olive oil and ethyl alcohol CME, and the recovery calculated on the basis of the results obtained by the proposed method. The recovery (R) was calculated as $R = 100 \times \{(Cspike − Csample)/Cstandard\}$, where Cspike is the concentration in the spiked CME sample, Csample is the concentration in the CME sample without spiking, and Cstandard is the added concentration. The results are average recoveries ($n = 3$ each) of the investigated analytes.

2.6.6. Dilution integrity

Dilution integrity was demonstrated by spiking the blank at 2 and 4 times the HQC concentration (3 mg/mL and 6 mg/mL, respectively). The blank matrix was prepared as for standard solutions and quality control samples by diluting 50 μL of blank matrix (olive oil or ethyl alcohol) in 9950 μL of isopropanol. Six replicates per dilution factor were prepared and diluted with blank matrix. The concentrations were calculated by applying the dilution factor 2 and 4 against freshly prepared calibration curve.

2.6.7. Stability

The short-term stability was determined for LQC and HQC samples in mobile phase diluted samples (olive oil and ethyl alcohol) for 24 h at room temperature ($n = 3$). The freeze-thaw stability of the drug in processed samples at $−20\,°C$ was confirmed after three freeze-thaw cycles on successive days ($n = 3$). Stability of the drugs in processed samples was assessed after 24 h in the autosampler at $10\,°C$ ($n = 3$). The drugs were considered stable if the mean concentration was within $±15\%$ of the nominal concentration.

2.7. Extraction methodologies

2.7.1. Olive oil and ethyl alcohol extracts (CMEs)

All CMEs and CMERs (olive oil and ethyl alcohol extracts of Bediol®) were at Farmacia Tundo Dr. Alfredo as specifically requested by the prescription.

2.7.1.1. Extracts prepared under refluxing (CMERs).

2.7.1.1.1. Olive oil. 2 g of Bediol® inflorescence, fine powder, were placed in 20 mL of olive oil in a round bottom flask with a condenser and heated at $110\,°C$ under magnetic stirring for 2 h 100 μL aliquot were sampled at selected time points starting from t_0 (time when the mixture reached $110\,°C$). The mixture was stirred at $110\,°C$ for 2 h and gradually cooled down to room temperature over at least 2 h. Subsequently, the mixture was paper filtered to obtain the final CMER.

2.7.1.1.2. Ethyl alcohol. 2 g of Bediol® inflorescence, fine powder, were placed in 20 mL of ethyl alcohol 96% into a round bottom flask and refluxed under magnetic stirring for 2 h 100 μL aliquot were sampled at selected time points starting from t_0 (time when the mixture starts to boil). Subsequently, the mixture was left to cool down to room temperature, then paper filtered and the solvent removed under vacuum. The residue was reconstituted with 20 mL of ethyl alcohol 96% and/or olive oil to obtain the final CMER.

2.7.1.2. Extracts prepared without refluxing (CMEs).

2.7.1.2.1. Olive oil. 5 g of cannabis inflorescence was finely grinded and added to 50 mL of pre-heated ($110\,°C$) olive oil. The mixture was stirred at $110\,°C$ for 2 h and then gradually cooled down to room temperature over at least 2 h. Subsequently, the mixture was paper filtered to obtain the final CME as requested by the prescription.

2.7.1.2.2. Ethyl alcohol. 5 g of cannabis inflorescence was finely grinded and added to 200 mL of ethyl alcohol 96%. The mixture was stirred at room temperature for at least 3 h. Subsequently, the mixture was paper filtered and the solvent removed under vacuum. The residue was reconstituted with 50 mL of ethyl alcohol 96% and/or olive oil to obtain the final CME as requested by the prescription.

All the samples were stored at $−80\,°C$ until analysis. 50 μL aliquot of olive oil or ethyl alcohol CMER and CME were dissolved in 2-propanol to the final volume of 10 mL 100 μL of the solution was diluted in 890 μL of mobile phase and 10 μL of IS (500 μg/mL) were added. The solution was vortex-mixed for 5 min and 5 μL were injected directly into the HPLC system.

2.7.2. Supercritical phase CO_2 extracts

Cannabis inflorescence was finely grinded and up to 20 g of plant material were loaded into the extraction tube. The extraction was carried out with a supercritical phase CO_2 extractor (OCO Labs Inc., mod. SuperC, Redway, CA) equipped with an automatic controller. The apparatus was pre-heated at $60\,°C$ and the working pressure was set at 3200 psi for 90 min. The extract obtained was reconstituted with 200 mL ethyl alcohol 96% and/or olive oil as requested by the prescription.

All the samples were stored at $−80\,°C$ until analysis. 50 μL aliquot of the extract were treated as for olive oil and ethyl alcohol extracts and injected directly into the HPLC system.

2.8. Stability of cannabinoids in CMEs

The stability of CBD-A, CBD, CBN, THC and THC-A in olive oil and ethyl alcohol was assessed by analyzing an olive oil and an ethyl alcohol CME stored in an amber glass container at room temperature ($25\,°C$) and in a refrigerator ($8\,°C$) for 3, 5, 6 and 10 days. The results are the mean $±$ SD of three replicates.

3. Results and discussion

3.1. Optimization of chromatographic conditions

One aim of this work was to develop a simple and rapid analytical method for the quantitative determination of the principal cannabinoids (CBD-A, CBD, CBN, THC and THC-A) to be employed by a pharmacist and applied to routinary analyses of CMEs. Regarding the HPLC method, although there are other studies where cannabinoids have been analyzed, albeit from different material and matrices [20–22], the separation of cannabinoids in isocratic conditions still represents a challenging issue since they possess similar chemical-physical properties. To this end, the best performance for the determination of cannabinoids have been achieved employing C18 columns with gradient elution [18,19]. However, a few studies report their determination by HPLC in isocratic conditions, although this is always performed on plant material and often with long analysis time [28,29]. To the best of our knowledge, none of the reported analyses was applied to CMEs. In this study, the chromatographic performance of two columns, including one fully porous (Luna-C18, 75×4.6 mm I.D., 3 μm, Phenomenex, Torrance, California, US) and one fused-core (Poroshell 120 SB-C18, 50×2.1 mm I.D., 2.7 μm), was evaluated. It is noteworthy that the fused-core technology was applied to the analysis of cannabinoids for the first time in this work. The presence of formic acid (0.1%) in the mobile phase provides a better peak shape and improves the resolution, especially for the cannabinoids in the acid form. Given the better chromatographic performance (in terms of both resolution and sensitivity), a shorter analysis time (10 min *vs.* 12 min) and a considerable saving of solvent consumed, working at a flow rate

of 0.5 mL/min instead of 1.5 mL/min, Poroshell column was finally selected for this study.

3.2. Method validation

3.2.1. Identification of cannabinoids

3.2.1.1. LC-UV. UV (228 nm) chromatograms of olive oil blank matrix, spiked olive oil blank matrix with IS, spiked olive oil blank with CBD-A, CBD, CBN, THC and THC-A at the MQC level (5.0 ng) and authentic olive oil CME (from Bediol®) are shown in Fig. S1A. The retention times of IS, CBD-A, CBD, CBN, THC and THC-A were approximately 0.9, 2.2, 2.6, 4.5, 5.9 and 8.5 min, respectively, within 10 min run time (Table S1). The chromatograms revealed symmetrical and completely separated peaks without any interfering peak. Thereby, the chromatographic method is sufficient to isolate IS, CBD-A, CBD, CBN, THC and THC-A from the blank without any interfering blank peak.

3.2.1.2. LC–MS. Identification of each compound is detailed in Tables 1A and 1B. Extracted molecular ion chromatograms (EICs) are shown in Fig. S1B. MS and MS^2 spectra of IS and analytes under investigation are reported in Fig. S2 (panels A and B, respectively). No interfering peaks were found in different random blank matrix samples at the retention times of either IS, CBD-A, CBD, CBN, THC or THC-A.

3.2.2. Specificity and selectivity

3.2.2.1. LC-UV. A peak purity test was performed in order to ensure that each peak corresponded to a single analyte. The overlap of extracted UV spectra at the start and at the end of the chromatographic peaks corresponding to IS, CBD-A, CBD, CBN, THC and THC-A showed a perfect match indicating that each peak is composed of a single compound (Fig. S3).

3.2.2.2. LC–MS. The peak purity test was carried out by overlapping the mass scan at the start and at the end of the chromatographic peaks (data not shown). The results indicated that each peak corresponded to a single analyte. The presence of a peak with m/z 347.0222 was observed at the retention time of 2.5 min in the chromatogram of a standard solution of CBD stored in methanol. The peak was not observed in the standard solution of CBD stored in ethanol. The molecular formula of the unknown compound suggested by the MassHunter software is $C_{21}H_{30}O_4$ with a score greater than 98% based on the difference in ppm of the molecular ion and on the isotopic pattern.

3.2.3. Linearity

3.2.3.1. LC-UV. As shown in Table S2A, linear calibration curves were obtained where the coefficient of determination (R^2) was greater than 0.998 over the pre-specified concentration range of 0.50–50.0 ng for CBD-A, CBD, CBN, THC and THC-A in three matrices, olive oil, ethyl alcohol and mobile phase. No significant difference was observed in calibration curves obtained in the three matrices employed ($p > 0.05$ with one-way ANOVA) indicating that matrix exerts no effect on area response employing UV detection (Fig. S4A). A signal-to-noise ratio greater than 3:1 was obtained for a concentration of 0.25 ng that was set as LOD. A signal-to-noise ratio greater than 10:1 was obtained for a concentration of 0.50 ng that was set as LOQ.

3.2.3.2. LC–MS. Table S2B shows the linear calibration curves that were obtained with a coefficient of determination (R^2) greater than 0.997 over the pre-specified concentration range of 0.50–50.0 ng for the analytes under investigation in olive oil, ethyl alcohol and mobile phase. As for LC-UV, no significant difference ($p > 0.05$ with one-way ANOVA) was observed between the three matrices

employed indicating that matrix exerts no effect on area response by MS detection (Fig. S4B). The concentrations 0.25 and 0.50 ng were set as LOD and LOQ respectively.

3.2.4. Precision (RSD) and accuracy

3.2.4.1. LC-UV. Table S3A shows in details the results obtained for the intra- and inter-day precision (RSD) and accuracy in olive oil and ethyl alcohol by LC-UV analysis. Intra- and inter-day RSD were within the acceptance limit (below 15%) for CBD-A, CBD, CBN, THC and THC-A in both matrices.

The intra-day accuracy for the cannabinoids under investigation was in the range of 93.52–103.3% in olive oil and 96.36–104.5% in ethyl alcohol. The inter-day accuracy was in the range of 92.36–105.1% in olive oil and 94.95–108.0% in ethyl alcohol. One can infer that precision and accuracy were within the acceptable limits.

3.2.4.2. LC–MS. Table S3B reports the results obtained for the intra- and inter-day precision (RSD) and accuracy in olive oil and ethyl alcohol by LC–MS. As expected, the results obtained by LC–MS were comparable to those obtained by LC-UV. Intra- and inter-day RSD were within the acceptance limit (below 15%) for CBD-A, CBD, CBN, THC and THC-A in both matrices.

The intra-day accuracy ranged from 90.04% to 114.3% in olive oil and from 93.33% to 105.6% in ethyl alcohol. The inter-day accuracy ranged from 95.20% to 106.2% in both olive oil and ethyl alcohol. As for LC-UV, LC–MS results indicated that precision and accuracy were within the acceptable limits.

3.2.5. Recovery

3.2.5.1. LC-UV. Mean recoveries ($n = 3$) were evaluated at LQC (0.5 ng), MQC (5.0 ng) and HQC (15 ng) level. The recovery was greater than 85% for all the cannabinoids under investigation in olive oil and greater than 93% in ethyl alcohol (detailed results are reported in Table S4A).

3.2.5.2. LC–MS. Table S4B shows that the results obtained by LC–MS perfectly matched with those obtained by LC-UV. Indeed, the recovery was greater than 85% for all the cannabinoids under investigation in olive oil and greater than 93% in ethyl alcohol.

3.2.6. Dilution integrity

3.2.6.1. LC-UV. The accuracies were found in the range 95–105%. The RSD was below 1% for all analytes in both matrices. Since accuracy and precision were within ±15% of the nominal concentration, the results met the EMEA acceptance criteria (Table S5A), revealing that samples with concentrations greater than the ULOQ could be re-analyzed by appropriate dilution.

3.2.6.2. LC–MS. Table S5B shows that the accuracies were in the range 92–102% by LC–MS. The RSD was below 8% for THC in olive oil and below 4% for all the other analytes in both matrices. The results met the EMEA acceptance criteria (Table S5B) as accuracy and precision were within ±15% of the nominal concentration. Therefore, samples with concentrations greater than the ULOQ could be re-analysed by appropriate dilution.

3.2.7. Stability

3.2.7.1. LC-UV. The stability of the analytes was evaluated at LQC (0.5 ng) and HQC (15 ng) level of samples prepared in olive oil and ethyl alcohol. Regarding short-term stability, olive oil and ethyl alcohol processed samples were stable after 24 h since accuracy% of both QCs was within the acceptable limits, *i.e.* 85–115%. The results showed that the analytes in olive oil and ethyl alcohol processed samples were stable after 24 h in autosampler at 10 °C. Accuracies were within the acceptable limits after three freeze-thaw cycles

Table 1A
Cannabinoids concentration at 0, 60 and 120 min of extraction in olive oil and ethyl alcohol CMEs analysed by LC-UV. Extraction performed under refluxing. The values are expressed in mg/mL as mean ± SD (n = 3).

Time (min)	Solvent	CBD-A	CBD	CBN	THC	THC-A
0	Olive oil	1.20 ± 0.01	0.32 ± 0.04	<LOQ	0.31 ± 0.03	1.05 ± 0.00
	Ethyl alcohol	6.25 ± 0.68	2.09 ± 0.12	<LOQ	1.53 ± 0.41	6.24 ± 0.67
60	Olive oil	3.24 ± 0.07	1.88 ± 0.10	<LOQ	1.53 ± 0.20	3.50 ± 0.88
	Ethyl alcohol	5.61 ± 0.40	3.11 ± 0.06	<LOQ	2.49 ± 0.05	7.18 ± 0.09
120	Olive oil	3.63 ± 1.26	2.69 ± 0.15	<LOQ	2.12 ± 1.13	3.78 ± 1.20
	Ethyl alcohol	4.59 ± 0.12	4.36 ± 0.01	<LOQ	2.65 ± 0.04	4.89 ± 0.02

Table 1B
Cannabinoids concentration at 0, 60 and 120 min of extraction in olive oil and ethyl alcohol CMEs analysed by LC–MS. Extraction performed under refluxing. The values are expressed in mg/mL as mean ± SD (n = 3).

Time (min)	Solvent	CBD-A	CBD	CBN	THC	THC-A
0	Olive oil	1.19 ± 0.12	0.38 ± 0.21	<LOQ	0.30 ± 0.02	1.07 ± 0.07
	Ethyl alcohol	5.89 ± 0.82	2.02 ± 0.15	<LOQ	1.52 ± 0.02	6.12 ± 0.74
60	Olive oil	2.90 ± 1.14	1.92 ± 0.16	<LOQ	1.47 ± 0.34	3.58 ± 1.41
	Ethyl alcohol	5.17 ± 0.34	3.03 ± 0.14	<LOQ	2.28 ± 0.17	6.94 ± 0.24
120	Olive oil	3.50 ± 0.30	2.76 ± 0.44	<LOQ	2.16 ± 1.01	4.25 ± 1.53
	Ethyl alcohol	4.40 ± 0.28	4.13 ± 0.21	<LOQ	2.48 ± 0.28	4.76 ± 0.21

Table 2
Cannabinoids concentration in olive oil, ethyl alcohol and supercritical phase CO_2 CMEs by HPLC-UV. Samples 1–4: Olive Oil Bediol® Extracts; samples 5–7: ethyl alcohol Bediol® extracts; samples 8–10: prepared by CO_2 supercritical phase extraction of Bediol®. The value are expressed in mg/mL as mean ± SD (n = 3).

Sample	CBD-A	CBD	CBN	THC	THC-A
1	6.02 ± 0.04	0.72 ± 0.08	<LOQ	1.11 ± 0.16	2.99 ± 0.12
2	7.00 ± 0.52	1.01 ± 0.26	<LOQ	1.46 ± 0.16	3.46 ± 0.24
3	6.01 ± 0.17	0.82 ± 0.17	<LOQ	1.41 ± 0.18	3.12 ± 0.58
4	6.44 ± 0.08	0.99 ± 0.15	<LOQ	1.43 ± 0.05	3.19 ± 0.17
5	5.77 ± 0.02	0.62 ± 0.21	<LOQ	1.29 ± 0.02	2.55 ± 0.45
6	5.86 ± 0.16	0.93 ± 0.04	<LOQ	1.53 ± 0.13	3.20 ± 0.02
7	5.31 ± 0.10	0.89 ± 0.15	<LOQ	1.39 ± 0.06	3.51 ± 0.35
8	5.22 ± 0.15	1.39 ± 0.03	<LOQ	1.16 ± 0.04	1.81 ± 0.05
9	4.67 ± 0.38	1.49 ± 0.06	<LOQ	0.90 ± 0.08	3.20 ± 0.23
10	4.57 ± 0.04	1.69 ± 0.06	<LOQ	1.10 ± 0.08	3.40 ± 0.05

indicating that analytes in olive oil and ethyl alcohol processed samples were stable under experimental conditions (Table S6A).

3.2.7.2. LC–MS. The results on the stability of the analytes evaluated at LQC (0.5 ng) and HQC (15 ng) level of samples prepared in olive oil and ethyl alcohol by LC–MS were identical to those obtained by LC-UV. Accuracies were within the acceptable limits for bench-top stability (24 h) in olive oil and ethyl alcohol processed samples. The analytes in the two matrices were stable after 24 h in autosampler at 10 °C and after three freeze-thaw cycles, indicating that analytes in olive oil and ethyl alcohol processed samples were stable under experimental conditions (Table S6B).

3.3. Extraction methodologies

The main goal of this work was to determine the concentration of the principal cannabinoids (CBD-A, CBD, CBN, THC and THC-A) in CMEs prepared in the pharmacy. To this end, three types of extraction methodologies of Bediol® were considered and compared: olive oil, ethyl alcohol and supercritical CO_2 CMEs. All CMEs were prepared by Farmacia Tundo Dr. Alfredo and analyzed by HPLC-UV. Moreover, an alternative method is herein presented for the preparation of olive oil and ethyl alcohol CMEs (CMERs), which were analyzed by LC-UV, LC–MS and GC–MS. This extraction methodology consisted of heating the mixture (inflorescence and solvent) under refluxing to prevent the evaporation of the volatile component of the plant material, such as terpenes, since recent studies have suggested their importance for the pharmacological activity of CMEs [2,10,25]. The analysis conducted on these extracts

revealed also important information on cannabinoids extraction and conversion rate.

3.3.1. Olive oil and ethyl alcohol extracts
3.3.1.1. Cannabinoids concentration in CMERs. The concentrations obtained by UV (Table 1A) and MS (Table 1B) detection were comparable. It is worth noting that a higher concentration of cannabinoids (CBD-A, CBD, THC and THC-A) was reached in a shorter time (60 min) when the extraction was performed in ethyl alcohol compared to olive oil, where the extraction of the acid cannabinoids still occurred after 120 min. Nonetheless, the concentrations in the two solvents were comparable after 120 min. The levels of CBN were always below the LOQ indicating low decomposition rate of THC. The proposed method could be applied to the routinary preparation of CMEs since it is simple and less time consuming than that generally employed by pharmacists.

3.3.1.2. Cannabinoids concentration in CMEs. Interestingly, the extraction rate of total CBD (intended as the sum of CBD-A and CBD) was higher in olive oil (about 78%) compared to ethyl alcohol, whereas the extraction rate of total THC was comparable in the two solvents (about 61%). It is also noteworthy that the extraction rate of both total CBD and THC in CMERs was much higher with respect to the CMEs, indicating that the proposed method could improve the preparation of CME prescriptions in terms of cannabinoid extraction yield. Moreover, the conversion rate of CBD-A to CBD in CMEs was dramatically lower compared to both olive oil and ethyl alcohol CMERs. On the other hand, THC-A conversion rate to THC was only slightly higher in CMERs. The concentration of each cannabinoid is reported in Table 2.
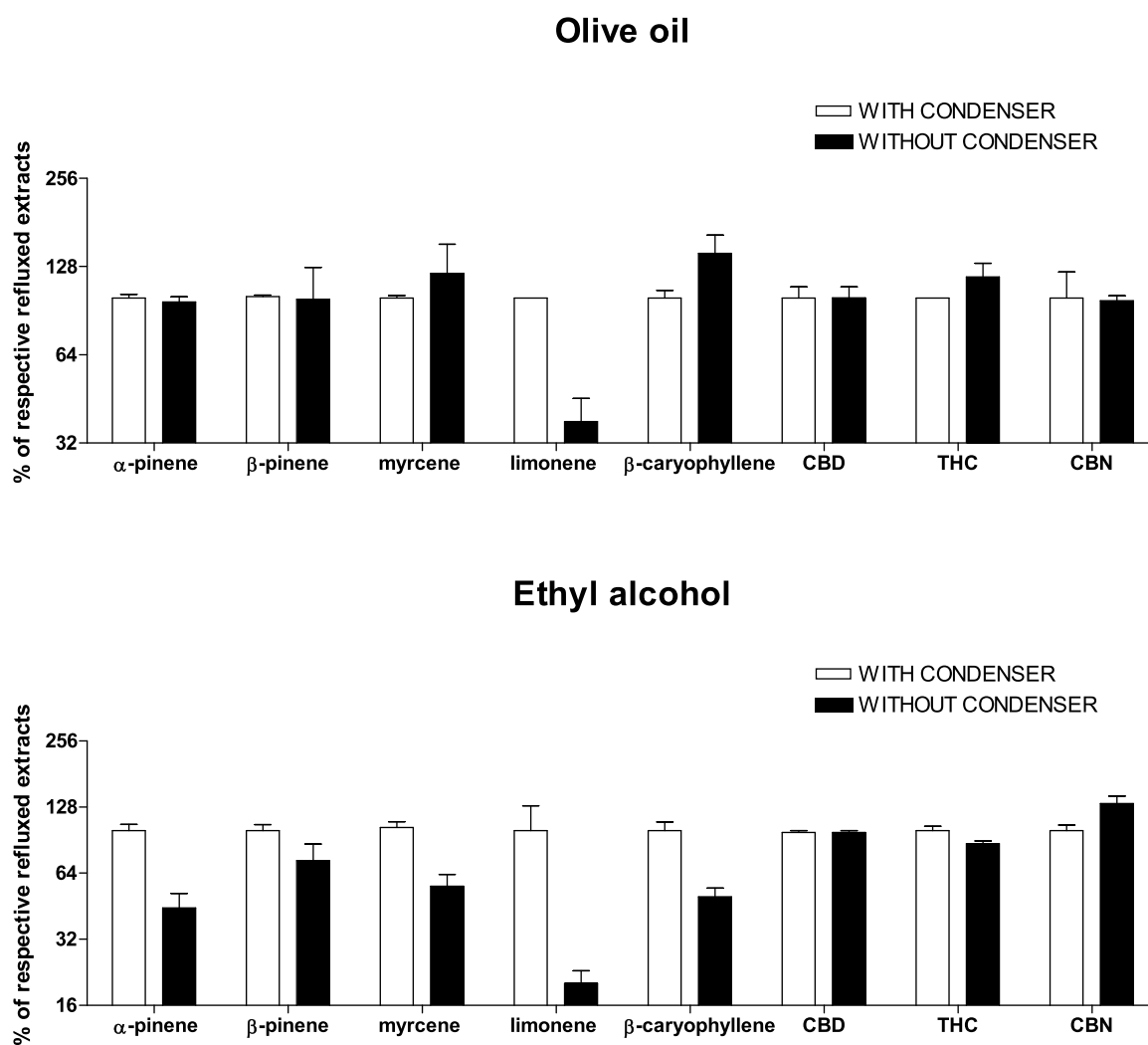
Olive oil

Ethyl alcohol

Fig. 2. Semi-quantitative analysis of terpene (α-pinene, β-pinene, myrcene, limonene and β-cariophyllene) and cannabinoid (CBD, THC and CBN) concentration in olive oil and ethyl alcohol CMEs. The values are percentage of respective concentrations in the refluxed extracts.

Table 3
Stability of CBD-A, CBD, THC and THC-A in olive oil and ethyl alcohol CMEs stored for 10 days at 8 and 25 °C. Values are expressed as percentage of the original concentration as mean ± SD (n = 3).

Days	Temperature (°C)	CBD-A		CBD		THC		THC-A	
		Olive oil	Ethyl alcohol	Olive oil	Ethyl alcohol	Olive oil	Ethyl alcohol	Olive oil	Ethyl alcohol
0	8	100 ± 1.16	100 ± 2.32	100 ± 0.47	100 ± 3.00	100 ± 7.09	100 ± 3.03	100 ± 5.24	100 ± 5.84
	25	100 ± 1.16	100 ± 2.32	100 ± 0.47	100 ± 3.00	100 ± 4.58	100 ± 3.03	100 ± 8.26	100 ± 6.12
3	8	98.4 ± 1.99	97.2 ± 3.20	98.4 ± 2.95	98.1 ± 3.01	99.5 ± 5.29	94.0 ± 7.52	94.5 ± 4.32	97.0 ± 6.27
	25	86.8 ± 2.56	69.9 ± 2.17	90.0 ± 2.00	51.4 ± 5.56	70.5 ± 5.47	27.8 ± 6.11	87.4 ± 6.09	61.3 ± 5.28
5	8	96.8 ± 3.59	94.8 ± 2.39	98.1 ± 2.00	96.2 ± 3.43	99.1 ± 5.34	88.1 ± 5.54	97.1 ± 5.96	93.9 ± 5.48
	25	82.8 ± 2.45	57.0 ± 2.53	88.7 ± 2.53	42.0 ± 6.93	59.4 ± 7.19	18.5 ± 6.11	82.0 ± 7.74	50.9 ± 4.55
6	8	96.1 ± 4.00	94.4 ± 2.34	97.4 ± 2.95	94.2 ± 3.71	99.1 ± 6.28	86.0 ± 6.22	95.8 ± 5.02	92.0 ± 4.57
	25	81.1 ± 2.21	51.7 ± 2.63	87.0 ± 1.94	39.1 ± 4.22	57.7 ± 6.22	17.9 ± 4.27	81.5 ± 6.55	46.3 ± 4.42
10	8	95.1 ± 3.72	88.2 ± 2.37	94.9 ± 2.85	85.8 ± 3.97	98.5 ± 7.65	77.5 ± 5.64	94.6 ± 6.24	84.1 ± 5.67
	25	80.1 ± 2.18	38.8 ± 2.30	86.2 ± 2.54	29.5 ± 5.50	56.6 ± 4.74	17.9 ± 5.36	78.0 ± 4.27	33.4 ± 4.14

3.3.2. Supercritical phase CO_2 CMEs

Supercritical phase CO_2 extraction of cannabinoids is still not well documented in the literature. Nonetheless, it is a promising alternative technique since there are no flammability or toxicity issues, solvent removal is simple and efficient, and the extract quality can be controlled by tuning the pressure and temperature [30]. Table 2 shows the concentration of the principal cannabinoids in CMEs obtained by supercritical phase CO_2. The extraction rate of total CBD with supercritical phase CO_2 was comparable to

that observed in ethyl alcohol (about 69% with respect to the content given by the certificate of analysis provided by the Bedrocan BV company), whereas the extraction rate of total THC was only slightly lower than that in olive oil and ethyl alcohol CMEs (57% vs. 61%). It is worth noting that CBD-A conversion rate to CBD was higher when the extraction was performed in supercritical phase CO_2 with respect to olive oil and ethyl alcohol; on the contrary, supercritical phase CO_2 CMEs registered the lowest conversion rate of THC-A to THC. This is probably due to different conversion kinet-
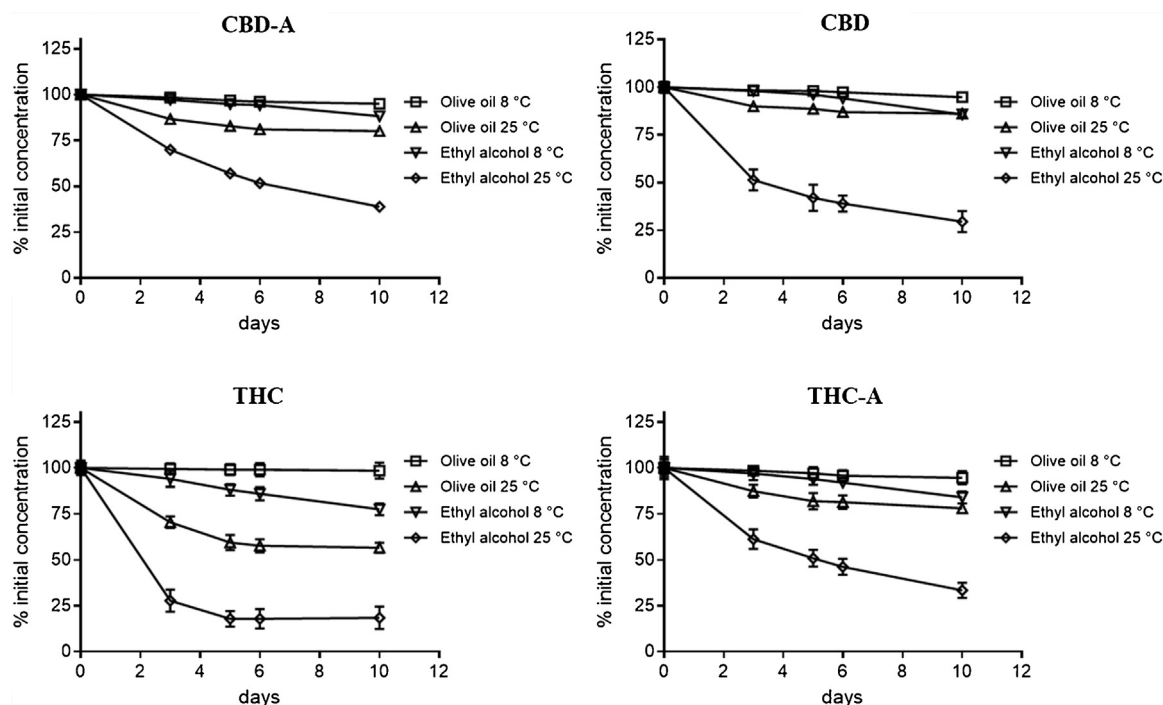
Fig. 3. Stability of cannabinoids in olive oil and ethyl alcohol CMEs at 8 and 25 °C after 3, 5, 6 and 10 days by LC-UV analysis.

ics of CBD-A and THC-A, which most likely depend on temperature and pressure.

3.4. GC–MS analysis

Since terpenes are not detectable by liquid chromatography, for a semi-quantitative evaluation of this class of compounds a GC–MS analysis was performed on both CMERs and CMEs. The area of the peaks in the CMEs were compared to those obtained in CMERs. The identity of the peaks α-pinene, β-pinene, myrcene, limonene, β-cariophyllene, CBD, THC and CBN could be unambiguously assigned since their retention times and mass spectra were compared to those of authentic standards and of NIST database. Since no derivatization was used, the high temperature that is applied in GC caused the decarboxylation of CBD-A and THC-A to their corresponding neutral form CBD and THC. Hence, it was not possible to distinguish between cannabinoids in the acid form and the corresponding neutral species. The results obtained suggested that whilst the amount of cannabinoids and sesquiterpene β-cariophyllene were comparable, the levels of low-boiling monoterpenes were several times higher in ethyl alcohol CMERs (Fig. 2). No significant difference between the two procedures was observed in terpene and cannabinoid concentration when the extraction was carried out in olive oil with the exception of limonene (Fig. 2). These data confirmed the results previously obtained by Romano and Hazekamp, which reported the largest terpene recovery in olive oil [19].

The GC–MS analysis confirmed and complemented the results obtained by LC-UV and LC–MS. Since it has been reported that terpenes are important for the pharmacological activity of CMEs because of their synergistic action with cannabinoids [2,10,25], the proposed extraction methodology is suitable for the preservation of terpenoids and other volatile compounds.

3.5. Stability of cannabinoids in CMEs

Fig. 3 shows the stability of the cannabinoids under investigation in both olive oil and ethyl alcohol 96% CMEs at two temperatures,

8 °C and 25 °C. The analyses were performed by HPLC-UV-DAD as described above. As expected, CMEs stored in the refrigerator at 8 °C were more stable than those kept at room temperature. The decrease of cannabinoids concentration at 8 °C was within 15% for CBD-A, CBD and THC-A after 10 days, whereas THC concentration decreased by about 23%. Surprisingly, ethyl alcohol 96% caused a more rapid decomposition of the cannabinoids with respect to olive oil, where the loss did not exceed 20% of their initial concentration for CBD-A, CBD and THC-A even after 10 days at room temperature. On the other hand, THC decomposed more rapidly compared to the other cannabinoids; indeed, its concentration loss at 25 °C was about 30% and 72% in olive oil and ethyl alcohol 96%, respectively, already after 3 days and about 44% and 82% in olive oil and ethyl alcohol 96%, respectively, after 10 days. The results are detailed in Table 3.

4. Conclusions

An LC-UV and LC–MS method has been developed for the qualitative and quantitative determination of the principal cannabinoids CBDA, CBD, CBN, THC and THCA. Both UV-DAD and QTOF detectors have been applied to the determination of cannabinoids in olive oil and ethyl alcohol cannabis-based medicinal extracts (CMEs), providing similar results and suggesting the equivalence of the two detection systems. The method developed was fully validated according to EMEA guidelines, suggesting that any pharmacist could analyze his own CMEs by simply employing the LC-UV system. Moreover, stability studies were conducted to evaluate the influence of the solvent (ethyl alcohol and olive oil) on cannabinoids degradation rates. The cannabis-based olive oil extracts preserve the cannabinoids concentration for longer time than ethyl alcohol ones. In either solvent, cannabinoids decomposition rate is very low for at least 10 days if the preparation is stored at 8 °C. An alternative cannabinoids extraction methodology has been proposed for both olive oil and ethyl alcohol CMEs (CMERs) and the results suggest that it could improve CMEs preparation in terms of cannabinoid extraction yield and conversion rate. Since this

methodology involves the heating of the plant material in the solvent under reflux, it allows to preserve most of the volatile compounds present in cannabis inflorescences, such as terpenes, which have been proved to exert a synergistic action with cannabinoids in the pharmacological activity of CMEs. A semi-quantitative analysis of this class of compounds was carried out by GC–MS, which confirmed that their concentration is much higher when extracted under refluxing.

Conflict of interest

The authors declare no competing financial interest.

Funding

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2016.05.033.

References

[1] M.A. Ware, K.R. Muller-Vahl, D. Abrams, F. Grotenhermen, A. Hazekamp, The medicinal use of cannabis and cannabinoids–an international cross-sectional survey on administration forms, J. Psychoact. Drugs 45 (2013) 199–210.

[2] J.M. McPartland, E.B. Russo, Cannabis is more than simply $\Delta 9$-tetrahydrocannabinol, Psychopharmacology 165 (2003) 431–432.

[3] K.S. Scott, S.L. Kacinko, B.C. Presley, B.K. Logan, S.M.R. Gurney, Pharmacology toxicology, and adverse effects of synthetic cannabinoid drugs, Forensic Sci. Rev. 26 (2014) 53–78.

[4] P. Robson, H. House, P. Makela, J. Aram, D.T. Wade, A preliminary controlled study to determine whether whole-plant cannabis extracts can improve intractable neurogenic symptoms, Clin. Rehabil. 17 (2003) 21–29.

[5] P. Robson, M. Ho, R.W. Jubb, C.S. McCabe, D.R. Blake, Preliminary assessment of the efficacy, tolerability and safety of a cannabis-based medicine (Sativex) in the treatment of pain caused by rheumatoid arthritis, Rheumatology 45 (2006) 50–52.

[6] J.C.M. Brust, T. Fife, J. Bronstein, S. Youssof, G. Gronseth, D. Gloss, B.S. Koppel, Systematic review: efficacy and safety of medical marijuana in selected neurologic disorders, Neurology 82 (2014) 1556–1563.

[7] K.L. Franson, A.M. Nussbaum, S. Wang, L.M. Borgelt, The pharmacologic and clinical effects of medical cannabis, Pharmacotherapy 33 (2013) 195–209.

[8] R.F. Wolff, S. Deshpande, M. Di Nisio, S. Duffy, A.V. Hernandez, J.C. Keurentjes, S. Lang, K. Misso, S. Ryder, S. Schmidlkofer, M. Westwood, J. Kleijnen, P.F. Whiting, Cannabinoids for medical use: a systematic review and meta-analysis, J. Am. Med. Assoc. 313 (2015) 2456–2473.

[9] E.B. Russo, Cannabinoids in the management of difficult to treat pain, Ther. Clin. Risk Manag. 4 (2008) 245–259.

[10] E.B. Russo, Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects, Br. J. Pharmacol. 163 (2011) 1344–1364.

[11] K.R. Müller-Vahl, F. Grotenhermen, The therapeutic potential of cannabis and cannabinoids, Dtsch. Arztebl. Int. 109 (2012) 495–501.

[12] W. Cely, S.A. Turkanis, R. Karler, The anticonvulsant activity of cannabidiol and cannabinol, Life Sci. 13 (1973) 1527–1531.

[13] H.B. Vassar, L.C. Knobloch, R.D. Sofia, Comparative analgesic activity of various naturally occurring cannabinoids in mice and rats, Psyehopharmacologia (Berl.) 40 (1975) 285–295.

[14] G. Ulrich-Merzenich, H. Wagner, Synergy research: approaching a new generation of phytopharmaceuticals, Phytomedicine 16 (2009) 97–110.

[15] I. Shirakawa, N. Kasinski, A. Pfeferman, E.A. Carlini, I.G. Karniol, Cannabidiol interferes with the effects of $\Delta 9$-tetrahydrocannabinol in man, Eur. J. Pharmacol. 28 (1974) 172–177.

[16] K. Misawa, I. Yamamoto, K. Watanabe, S. Takeda, Cannabidiolic acid as a selective cyclooxygenase-2 inhibitory component in cannabis, Drug Metab. Dispos. 39 (2008) 1917–1921.

[17] Z. Mehmedic, T.P. Murphy, M.A. ElSohly, S.A. Ross, GC–MS analysis of the total $\Delta 9$-THC content of both drug- and fiber-type cannabis seeds, J. Anal. Toxicol. 24 (2000) 715–717.

[18] R. Verpoorte, T.J. Raharjo, Methods for the analysis of cannabinoids in biological materials: a review, Phytochem. Anal. 15 (2004) 79–94.

[19] A. Hazekamp, L. Romano, Cannabis oil: chemical evaluation of an upcoming cannabis-based medicine, Cannabinoids 1 (2013) 1–11.

[20] L. Tedeschi, G. Frison, F. Castagna, S.D. Ferrara, Solid-phase extraction and HPLC-UV confirmation of drugs of abuse in urine, J. Anal. Toxicol. 16 (1992) 217–222.

[21] E. Szwarc, A. Tracqui, B. Ludes, C. Jamey, Determination of cannabinoids in whole blood by UPLC–MS–MS, J. Anal. Toxicol. 36 (2012) 280–287.

[22] A. Scholera, J. Drewe, S.B. Grauwilera, Development of a LC/MS/MS method for the analysis of cannabinoids in human EDTA-plasma and urine after small doses of *Cannabis sativa* extracts, J. Chromatogr. B 850 (2007) 515–522.

[23] E.R. Heerdink, A. Hazekamp, The prevalence and incidence of medicinal cannabis on prescription in The Netherlands, Eur. J. Clin. Pharmacol. 69 (2013) 1575–1580.

[24] Ministero della Salute, GU Serie Generale, n. 279 del 30-11-2015.

[25] E.B. Russo, J.M. McPartland, Cannabis and cannabis extracts: greater than the sum of their parts? J. Cannabis Ther. 1 (2001) 103–132.

[26] EMEA ICH Topic Q2(R1) Validation of Analytical Procedures: Text and Methodology (2006).

[27] International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH), Guideline Q2(R1)-Validation of Analytical Procedures: Text and Methodology, ICH Secretariat, c/o IFPMA, Geneva (2005) p. 1.

[28] D.W. Wingard, A.N. Masoud, High performance liquid chromatography with electrochemical detection. 1: Separation of cannabis constituents and quantitation of $\Delta 9$ tetrahydrocannabinol, J. High Resolut. Chromatogr. 2 (1979) 118–122.

[29] J.C. Wong, A. Sabri, P.M. Fischer, D.A. Barrett, C.S. Constantinescu, P. Gershkovich, A. Zgair, Development of a simple and sensitive HPLC-UV method for the simultaneous determination of cannabidiol and $\Delta (9)$-tetrahydrocannabinol in rat plasma, J. Pharm. Biomed. Anal. 114 (2015) 145–151.

[30] H. Perrotin-Brunel, Dissertation: sustainable production of cannabinoids with supercritical carbon dioxide technologies (2011) uuid: c1b4471f-ea42-47cb-a230-5555d268fb4c.