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A Metabolomic Approach Applied to a Liquid Chromatography Coupled to High-Resolution Tandem Mass Spectrometry Method (HPLC-ESI-HRMS/MS): Towards the Comprehensive Evaluation of the Chemical Composition of Cannabis Medicinal Extracts

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ABSTRACT:

Introduction – *Cannabis sativa* L. is a powerful medicinal plant and its use has recently increased for the treatment of several pathologies. Nonetheless, side effects, like dizziness and hallucinations, and long-term effects concerning memory and cognition, can occur. Most alarming is the lack of a standardised procedure to extract medicinal cannabis. Indeed, each galenical preparation has an unknown chemical composition in terms of cannabinoids and other active principles that depends on the extraction procedure.

Objective – This study aims to highlight the main differences in the chemical composition of Bediol® extracts when the extraction is carried out with either ethyl alcohol or olive oil for various times (0, 60, 120 and 180 min for ethyl alcohol, and 0, 60, 90 and 120 min for olive oil).

Methodology.

Cannabis medicinal extracts (CMEs) were analysed by liquid chromatography coupled to high-resolution tandem mass spectrometry (LC–MS/MS) using an untargeted metabolomics approach. The data sets were processed by unsupervised multivariate analysis.

Results – Our results suggested that the main difference lies in the ratio of acid to decarboxylated cannabinoids, which dramatically influences the pharmacological activity of CMEs. Minor cannabinoids, alkaloids, and amino acids contributing to this difference are also discussed. The main cannabinoids were quantified in each extract applying a recently validated LC–MS and LC-UV method.

Conclusions – Notwithstanding the use of a standardised starting plant material, great changes are caused by different extraction procedures. The metabolomics approach is a useful tool for the evaluation of the chemical composition of cannabis extracts.

Additional Supporting Information may be found online in the supporting information tab for this article.

Keywords: cannabis medicinal extracts; cannabinoids extraction; metabolomics; mass spectrometry

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Introduction

Notwithstanding the large increase in the use of cannabis for therapeutic purposes in recent years (Blake *et al.,* 2006; Borgelt *et al.,* 2013; Koppel *et al.,* 2014; Whiting *et al.,* 2015), there is still the need for a standardised procedure for the preparation of cannabis medicinal extracts (CMEs) and very few scientific studies on the actual chemical composition of CMEs have been published (Romano and Hazekamp, 2013). Most of the existent research studies regard the determination of the main cannabinoids in plant materials (Stolker *et al.,* 2004; Hazekamp *et al.,* 2005; De Backer *et al.,* 2009). Over a hundred cannabinoids have been identified in different strains of *Cannabis sativa* L. The most abundant and most known are cannabidiol (CBD) and (−)-*trans*-Δ^9-tetrahydrocannabinol (THC), which are present in the acid form in the inflorescence, namely cannabidiolic acid (CBDA) and tetrahydrocannabinolic acid (THCA), respectively. Each cannabinoid possesses completely different pharmacological activities. For instance, the pharmacological use of CBDA and THCA has not been thoroughly disclosed to date as these acid forms of cannabinoids are recognised as pharmacologically inactive (Yamauchi *et al.,* 1967; Burstein, 1999). Nonetheless, there is evidence that they exert anti-proliferative/pro-apoptotic effects in a few cancer cell lines (Ligresti *et al.,* 2006). However, THC acts on CB_1 central receptors leading to the psychotropic and hallucinating effects, while CBD seems to have analgesic, antiepileptic and antioxidant activity and to reduce THC side effects (Russo, 2008; Grotenhermen and Muller-Vahl, 2012). The interest in the pharmacological activity of the other cannabinoids present in lower concentration in the inflorescence has recently grown. One example is cannabigerol (CBG), deriving from cannabigerolic acid (CBGA), the stem cell of all cannabinoids, which proved to relieve intraocular pressure (Colasanti, 1990).

The scientific literature reveals a serious gap concerning the determination of the concentration of the two main cannabinoids THC and CBD in CMEs. The first research work regarding the evaluation of the main cannabinoids concentration and stability in oil and ethyl alcohol CMEs by LC–MS (and LC-UV) was developed and validated by our group (Citti *et al.,* 2016a). More recently, Pacifici *et al.* () described another analytical method addressing the same issue in cannabis based oil and tea preparations.

It is known that both CBDA and THCA, as well as the other acid cannabinoids, do not possess any psychotropic activity mainly because the dissociation of the carboxylic acid moiety at physiological pH of 7.4 prevents the blood brain barrier crossing (Moreno-Sanz, 2016). Only a chemical transformation, which could be accelerated by heat, leads to the formation of the well-known cannabinoids CBD and THC. Moreover, it should be considered that CBDA and THCA are not converted when administered, but rather they exert their biological effect (anti-inflammatory, neuroprotective, anti-emetic, etc. (Bolognini *et al.,* 2013)) only peripherally. Practically, fresh untreated inflorescence would have a scarce psychotropic effect and only the heating would transform it into a "drug". This explains the reason why smoking is the preferential administration route for recreational purposes (Dussy *et al.,* 2005). Indeed, heating the inflorescence at high temperature causes the immediate decarboxylation of the cannabinoid THCA to get THC (and CBD from CBDA) (Taschwer and Schmid, 2015). After being absorbed through the lungs, in a few minutes THC can be found in the central nervous system (Huestis *et al.,* 1992). Moreover, the decarboxylation of THCA leads to the formation of THC and a small part of the latter oxidises to cannabinol (CBN), which is a cannabinoid with potent sedative properties.

Usually, CMEs dispensed by a pharmacist report the concentration of THC without specifying whether it refers to the carboxylated form or to the non-carboxylated one or even to the sum of the two. This strictly depends on the analytical method used to determine the concentration of the cannabinoid. Gas chromatography (GC) involves the heating of the sample above 200°C prior to the analysis, whereas liquid chromatography (LC) generally works at room temperature. Unless the sample is previously derivatised, unlike LC, GC does not distinguish between the two forms and gives the total THC content (Ambach *et al.,* 2014). If the extracts have not been properly heated, only the carboxylated non-psychoactive cannabinoid will be administered, with therapeutic indications completely different from those of the decarboxylated cannabinoid.

To further complicate the picture of the therapeutic effects of cannabis extracts, several scientific papers suggest the presence of other bioactive components in the plant inflorescence different from cannabinoids, such as terpenes, flavonoids, etc. These classes of compounds exert a pharmacological activity that is synergic with that of cannabinoids (McPartland and Russo, 2001; Russo and McPartland, 2003). In particular, terpenes are volatile and easily degradable. Therefore, different times and temperatures of extraction, as well as different solvents, affect the chemical composition of the final extracts.

The uncertainty in this scenario increases even more when talking about the stability of CMEs. The German Drug Codex (DAC), which is published by the Federal Union of German Associations of Pharmacists (ABDA) and functions as a supplement book to the pharmacopoeia, suggests a CBD oil preparation that is guaranteed as stable for 28 days, though admitting the lack of scientific studies on the integrity of the active principles over the time (DAC/NRF, 2015).

To date no standardised methodology is available for the preparation of CMEs with a known and uniform concentration of the main cannabinoids. The aim of the present study is to highlight the main changes in the chemical composition of the cannabis inflorescence when the extraction is carried out in different solvents and over various times. In fact, the main cannabinoids do not represent the only issue at this regard, but it is important to consider a plethora of other bioactive molecules, some of them with unknown biological activity, that are present in different concentrations in the CME depending on the extraction time. The present work reports the analysis of CMEs obtained in two solvents, ethyl alcohol and olive oil, and at various times: 0, 60, 120 and 180 min for ethyl alcohol CMEs and 0, 60, 90 and 120 min for olive oil CMEs. To this end, an untargeted metabolomics approach was used. Metabolomics is an "omics" study that can be used to acquire comprehensive information on the composition of a metabolite pool to provide a functional snapshot of a biological sample state at a molecular level. The comprehensive analysis of all metabolites within a biological sample is a very ambitious goal and is still far from a reality for any system, although substantial progress is being made (Sumner *et al.,* 2003). Indeed, in the past few years dramatic developments in high-throughput metabolomics have been achieved, especially due to the aid of bioinformatics technologies. In this work, a high-performance liquid chromatography coupled to tandem high-resolution mass spectrometry (HPLC-HRMS/MS) method has been employed for the metabolomic analysis of the selected CMEs. HPLC-HRMS/MS data were processed and analysed with a multi-group job by the XCMS Online web platform (Tautenhahn *et al.,* 2012) and the compounds with the most significant variations in concentration were identified using

authentic standards (CBDA, THCA, CBGA, CBD, THC, CBN, CBG, choline, arginine, and proline). A tentative identification of a few interesting compounds was hypothesised on the basis of accurate mass, isotopic pattern and high-resolution fragmentation spectrum match with METLIN (Zhu *et al.,* 2013) online database. Moreover, the main cannabinoids (CBDA, THCA, CBD, THC and CBN) concentration was evaluated in each extract using the recently validated LC–MS/MS method (Citti *et al.,* 2016a).

Experimental

Materials

Acetonitrile, water, 2-propanol, formic acid were all LC–MS grade and were purchased from Carlo Erba (Milan, Italy). Pharmaceutical grade refined olive oil was bought from Fagron Italia Srl (Bologna, Italy). Ethyl alcohol was of pharmaceutical grade bought from Carlo Erba (Milan, Italy). Arginine, choline and proline were from Sigma Aldrich (Milan, Italy). CBDA, THCA, CBGA, CBD, THC, CBN and CBG were purchased from Farmalab Srl, (Vado Ligure, Italy).

In this work there was no handling of new cannabis based material since all the samples employed were the same that have been previously analysed and reported in our recent paper (Citti *et al.,* 2016a).

Extraction methodology

The extraction procedure followed is the one recently proposed by Citti *et al.* (2016a), which involves the use of a condenser to minimise the loss of any volatile substance.

Extraction with ethyl alcohol

First, 2 g of Bediol® inflorescence (Bedrocan BV, Veendam, The Netherlands), fine powder, were placed in 20 mL of ethyl alcohol 96% into a round bottom flask and refluxed under magnetic stirring for 3 h (refluxing temperature 78°C). Then, 100 μL aliquot were sampled at t_0 (time when the mixture starts to boil) and three selected time points (60, 120 and 180 min). Subsequently, the mixture was left to cool down to room temperature, then paper filtered to obtain the final alcohol CME.

Extraction with olive oil

First, 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. Then, 100 μL aliquot were sampled at t_0 (time when the mixture reached 110°C) and at three selected time points (60, 90 and 120 min). 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 oil CME.

Sample preparation

For the metabolomics experiments, 50 μL aliquot of ethyl alcohol or olive oil CME at the selected time points were dissolved in 2-propanol to the final volume of 10 mL. Next, 100 μL of the solution was diluted in 890 μL of mobile phase and added of 10 μL of diazepam, used as internal standard (IS), at a final concentration of 50 μg/mL. The solution was vortex-mixed for 5 min. Then, 5 μL were injected directly into the HPLC system and analysed in five replicates. For the quantitative determination of CBDA, CBD, CBN, THC and THCA, 10 μL of IS was added at a final concentration of 500 μg/mL in line with the recently published LC–MS and LC-UV method (Citti *et al.,* 2016a).

For the metabolomics experiments, a pooled "quality control" (QC) sample was prepared by mixing equal volumes (20 μL) from each of the 24 CMEs (three samples of each of the four alcohol CMEs and three samples of each of the four oil CMEs) and used for method validation. Analysis of the QC sample was performed in triplicate at the beginning of each batch and every 15 runs.

LC-HRMS and LC-HRMS/MS method

HPLC analyses were performed on an Agilent Technologies (Santa Clara, CA, USA) modular model 1200 system, consisting of a vacuum degasser, a binary pump, a thermostated autosampler, a thermostated column compartment, a diode array detector (DAD) and a 6540 quadrupole time-of-flight (QTOF) mass analyser with an electrospray ionisation (ESI) source. A Poroshell column (Poroshell 120 EC-C18, 3.0 mm × 50 mm, 2.7 μm, Agilent, Milan, Italy) was used with a mobile phase composed of 0.1% (*v/v*) formic acid in both water (A) and acetonitrile (B). The analyses were carried out using an elution profile composed of a linear gradient from 5% to 95% B over 45 min; the mobile phase composition was held at 95% B for 10 min and then it was brought back to the initial composition (5% B) over 5 min and the column equilibrated for another 5 min. The flow rate was kept at 0.3 mL/min throughout the analytical run. The total run time was 65 min. The column temperature was set at 25°C. The sample injection volume was 5 μL. Five replicates were analysed for each sample. The HPLC-ESI-MS and MS/MS system operated in both positive (ESI+) and negative (ESI−) ionisation mode. The experimental parameters were set as follows: the capillary voltage was 3.5 kV, the nebuliser (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. The mass spectrometer was calibrated using the Agilent low-mass calibration mix. The calibration was run in both ESI+ and ESI− mode in the low-mass range of *m/z* 50–1700 at high resolution. The calibration was accepted if the parts per million (ppm) error was <0.5 ppm. 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 50–1700. MS/MS spectra were automatically performed with N_2 as the collision gas in the *m/z* range 50–1700, using the auto MS/MS function and a collision energy of 20 eV.

Cannabinoids concentrations (CBDA, THCA, CBD, THC and CBN) were determined in each extract employing a recently validated chromatographic method coupled to DAD and MS detector (Citti *et al.,* 2016a). The method involved an isocratic elution with water/acetonitrile 30:70 (*v/v*) and 0.1% formic acid (*v/v*) with a flow rate of 0.5 mL/min on the previously described Poroshell column. UV peaks were extracted at 228 nm (band width 4 nm). Extracted ion chromatograms (EICs) were obtained with an tolerance window of 10 ppm *m/z* from total ion chromatogram (TIC) employing the *m/z* corresponding to $[M + H]^+$ (285.0770 for IS, 359.2224 for CBDA and THCA, 315.2314 for CBD and THC, and 311.2000 for CBN) and $[M\text{-}H]^-$ of authentic standards (283.0644 for IS, 357.2017 for CBDA and THCA, 313.2027 for CBD and THC, and 309.1798 for CBN).

The elution order on the Poroshell C18 column was as follows: IS, CBDA, CBD, CBN, THC and THCA and their retention times in isocratic conditions were 0.9, 2.2, 2.6, 4.5, 5.9 and 8.5 min, respectively. In the gradient conditions (metabolomics experiments), the respective retention times were 22.7, 37.4, 38.6, 42.0, 43.7 and 46.0 min.

XCMS online data analysis and MetaboAnalyst results processing

Raw HPLC–MS/MS metabolomic data were converted to *mzXML* files using ProteoWizard MS Convert version 3.0.4146 (Chambers *et al.,* 2012). The *mzXML* files were uploaded to XCMS Online web platform for data processing, which applied peak detection, retention time correction with respect to the IS retention time, profile alignment, and isotope annotation. Data were processed as a multi-group design experiment, and the parameters were set as follows: centWave for feature detection ($\Delta m/z$ = 15 ppm, minimum and maximum peak width 10 and 120 s, respectively); obiwarp settings for retention time correction (profStep = 1); parameters for chromatogram alignment, including mzwid = 0.015, minfrac = 0.5, and bw = 5. The relative quantification of the detected features was based on EIC areas. The results output were exported by direct download from XCMS Online. Metabolite identification was based on accurate mass (within 5 ppm) and/or MS/MS

data match against MS/MS spectra available on METLIN. MS/MS data of CBDA, Δ^9-THCA, CBGA, CBD, Δ^9-THC, CBN, CBG, choline, arginine and proline were confirmed also via matching against authentic standards. XCMS *csv* file output was processed using Metaboanalyst 3.0 (Xia *et al.,* 2015), which implemented the statistical analysis and graphical representation (3D–PCA, heatmap, etc.). Principal component analysis (PCA) was performed after normalisation based on sum of peak areas and Pareto scaling. Heatmaps were built setting the following parameters: distance measure: Euclidean, clustering algorithm: Ward, standardisation: autoscaling features from normalised data.

Results and discussion

Extraction methodologies and sample preparation

One of the most commonly used extraction methodology of medicinal cannabis for galenical preparations consists of mixing the pulverised inflorescence with an appropriate volume of ethyl alcohol or olive oil in a 1:10 (*w/v*) ratio and heating it for a time and a temperature decided by the individual pharmacist. Therefore, it becomes important to evaluate how the chemical composition of the final CME is affected by various times of extraction. Indeed, the principal cannabinoids like CBD and THC are present in the inflorescence as carboxylated species and undergo decarboxylation with a rate strictly dependent on the solvent and temperature employed. It is important to take into account not only the extraction rate of the active principles of the plant material but also their decomposition and decarboxylation rates. Hence, a targeted metabolomics analysis is not suitable for a comprehensive characterisation of the chemical composition of CMEs. In this context, untargeted metabolomics would represent the analytical approach of choice for an accurate evaluation of the qualitative changes in bioactive substances, which are closely related to the aforementioned parameters. In contrast to a targeted metabolomics experiment, which measures ions from known compounds, an untargeted metabolomics experiment registers all ions within a certain mass range, which is closer to the aim of this work.

By performing the extraction in ethyl alcohol under refluxing, we set the temperature at 78°C. The extraction in olive oil was carried out at 110 and 145°C. Unfortunately, organoleptic properties of the cannabis based galenical preparations extracted in olive oil at the higher temperature (145°C) made it unsuitable for the patient compliance. Hence, the experiments performed at 145°C were discarded. The extractions were followed for 180 min in ethyl alcohol and 120 min in olive oil with selected time points (0, 60, 120 and 180 min for ethyl alcohol and 0, 60, 90 and 120 min for olive oil).

LC–MS and LC–MS/MS method

Several types of columns were tested for the metabolomic experiments on CMEs. Poroshell column was chosen due to its efficient chromatographic separation (Citti *et al.,* 2016b, 2016c); moreover, it provided a higher number of peaks compared to fully porous C18 stationary phase based columns. Moreover, UHPLC stationary phases, such as ZORBAX Eclipse Plus C18 (50 mm × 3 mm i.d., 1.8 μm, Agilent), were also evaluated with the result of high back-pressures and poor reproducibility.

On the basis of our research group's considerable experience in LC–MS and LC–MS/MS analysis (Carrozzo *et al.,* 2010, 2012, 2014; Cannazza *et al.,* 2012, 2014; Battisti *et al.,* 2016), we developed a chromatographic method exploiting the metabolomic approach. The mobile phase employed was water/acetonitrile with 0.1% formic acid (*v/v*) with a linear gradient from 5% to 95% acetonitrile in 45 min and a total run time of 65 min. The detectors used were a DAD operating in the range of 190 to 500 nm and a QTOF mass spectrometer equipped with an ESI operating in both positive and negative mode.

In order to guarantee the accuracy and reliability of raw data, LC–MS method validation was performed prior to the measurement of extract samples. A pooled QC sample was analysed three times at the beginning of the analytical run to ensure system equilibration and then once every five runs to provide robust quality assurance for each feature change detected. Five representative peaks corresponding to CBDA, THCA, CBD, THC and CBN in the chromatograms of QC samples detected in both ESI+ and ESI− mode were selected for method validation. Overall, the retention times and *m/z* of the five selected peaks in all the analysed HPLC chromatograms were precisely the same ($\Delta R_T < 0.1$ min, Δppm <0.3). The relative standard deviations (RSD%) of peak area were measured as less than 4% and less than 7% for ESI+ mode in ethyl alcohol and olive oil, respectively, and less than 6% and 8% for ESI− mode in ethyl alcohol and olive oil, respectively. The results of the method validation indicated good precision, stability and repeatability and

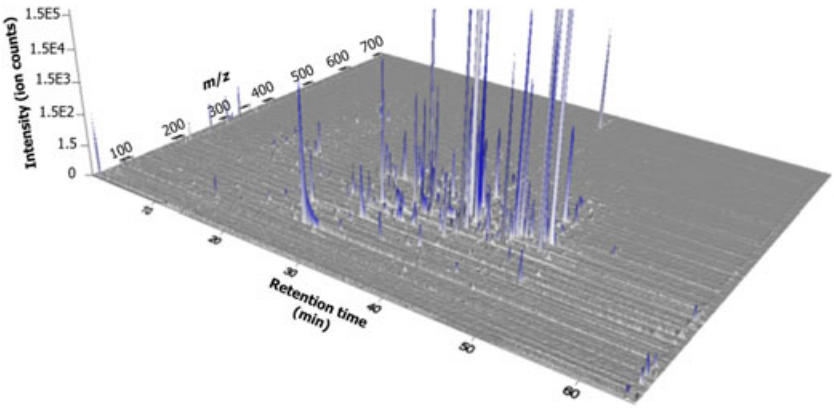
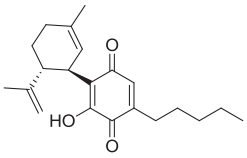
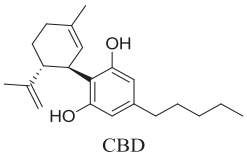
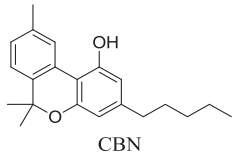
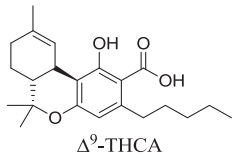
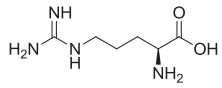


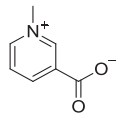
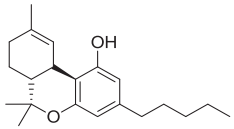
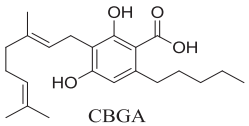
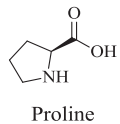
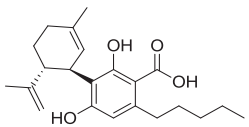
Figure 1. Three-dimensional (3D) total ion chromatogram (TIC) of an alcohol cannabis medicinal extract (CME) at 60 min in negative ionisation (ESI−) mode. The retention time (min) is represented on the *x* axis, the peak intensity (ion counts) on the *y* axis and the *m/z* values on the *z* axis. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1. Identification of the main cannabinoids and other metabolites in ethyl alcohol (alcohol) and olive oil (oil) cannabis medicinal extracts (CMEs). Where present, the fragmentation profile of the ion has been reported

Compound	Formula	$[M + H]^+ m/z$		$[M-H]^- m/z$		Solvent	R_T (min)[a]	Trend[b]	Level of ID[c]
		MS	MS/MS	MS	MS/MS				
 HU-331	$C_{21}H_{28}O_3$	329.2103 (43)[d] Δppm 2.5	311.1978 (29) 271.1685 (35) 193.1226 (100) 121.1016 (35)	327.1956 (7) Δppm 2.9	310.1876 (6) 218.0919 (9) 191.1053 (100)	alcohol oil	32.20 32.23	↑ NS	2
 CBD	$C_{21}H_{30}O_2$	315.2312 (97) Δppm 2.1	259.1737 (46) 193.1225 (100) 135.1173 (46)	313.2161 (54) Δppm 3.8	245.1522 (100) 179.1054 (43) 107.0491 (15)	alcohol oil	38.83 38.83	↑ ↑	3
 CBN	$C_{21}H_{26}O_2$	311.2037 (82) Δppm −0.5	293.1914 (38) 241.1239 (33) 223.1133 (100)	309.1848 (100) Δppm 3.9	279.145 (11)	alcohol oil	42.05 42.16	↑ ↑↓	3
 Δ^9-THCA	$C_{22}H_{30}O_4$	359.2212 (4) Δppm 1.3	341.2091 (100) 313.2133 (1) 219.1003 (13)	357.2081 (100) Δppm 2.7	339.2066 (58) 313.2244 (27) 245.1616 (31) 179.1116 (24)	alcohol oil	46.02 46.21	↓ ↑	3
 Arginine	$C_6H_{14}N_4O_2$	175.1177 (<5) Δppm 4.3	158.0923 (<5) 130.0950 (5) 116.0717 (6) 70.0656 (100) 60.0563 (31)	–	–	alcohol oil	0.90 0.88	↑ ↑↓	3

(*Continues*)

Table 1. *(Continued)*

Compound	Formula	$[M + H]^+ m/z$		$[M-H]^- m/z$		Solvent	R_T (min)[a]	Trend[b]	Level of ID[c]
		MS	MS/MS	MS	MS/MS				
 Trigonelline	$C_{22}H_{32}O_2$	138.0538 (100) Δppm 3.3	120.0446 (6) 94.0659 (80) 78.0348 (39) 65.0384 (45)	–	–	oil alcohol	0.97 0.94	↑ ↑↓	2
 Δ^9-THC	$C_{21}H_{30}O_2$	315.2319 (100) Δppm −0.1	259.1731 (43) 193.1251 (74) 135.1186 (35)	313.2167 (100) Δppm 1.9	295.2031 (6) 245.1504 (26)	alcohol oil	43.76 43.87	↑ ↑	3
 CBGA	$C_{22}H_{32}O_4$	361.2359 (5) Δppm 4.0	343.2265 (37) 261.1456 (7) 219.0992 (100) 175.1457 (10) 113.0594 (7)	359.2222 (43) Δppm 1.6	341.1968 (100) 326.1786 (76) 245.1407 (63) 227.1331 (75) 107.0415 (85)	alcohol oil	38.52 38.55	↓ ↑	3
 Proline	$C_5H_9NO_2$	116.0702 (<5) Δppm 3.5	101.9702 (<5) 80.9744 (<5) 70.0665 (100)	–	–	alcohol oil	0.96 0.94	↑ ↑↓	3
 CBDA	$C_{22}H_{30}O_4$	359.2213 (<1) Δppm 1.3	341.2143 (100) 313.2047 (<1) 219.1012 (15)	357.2057 (54) Δppm 4.0	340.2020 (23) 312.1282 (87) 227.1303 (100) 173.0881 (63) 107.0441 (93)	alcohol oil	37.59 37.63	↓ ↑	3
	$C_5H_{14}NO$	104.1074 (26) Δppm 1.3	77.0378 (<5) 60.0813 (100) 58.0657 (74)	–	–	alcohol oil	0.93 0.91	↑ ↑↓	3

[a] R_T = retention time.

[b] Trend: concentration trend over time. ↑ = concentration increases over time; ↓ = concentration decreases over time; NS (not significant) = concentration does not change.

[c] Level of identification: 1 = MS *m/z* match with online database; 2 = MS and MS/MS *m/z* match with online database; 3 = R_T and MS/MS *m/z* match with authentic standard.

[d] Figures in brackets are ion relative intensities in MS or MS/MS spectra.

suggested that the LC–MS conditions employed were suitable for untargeted metabolomics analysis.

Global profiling of alcohol and oil CMEs

The chemical composition of *C. sativa* L. extracts has already been explored in several studies, albeit only very few articles report their LC-HRMS profiling (Marti *et al.,* 2014). Most of the research work on the chemical composition of cannabis extracts has been conducted by NMR (Peschel and Politi, 2015; Wang *et al.,* 2016). Metabolomic studies were mainly focused on the different strains of *C. sativa* L. without any particular attention to the extraction methodology (Choi *et al.,* 2004; Fischedick *et al.,* 2010). Untargeted metabolomics allows to highlight different chemical profiles that could be correlated with different pharmacological activities. LC-HRMS has become the technique of choice for untargeted metabolomics studies, thus it was employed in this work to evaluate the effects of time and most used solvents for the extraction of medicinal cannabis. The three-dimensional (3D) chromatogram obtained with LC-HRMS in scan mode (Figure 1) highlights the vast chemical complexity of an alcohol CME and only a minor part of the compounds present in the extract can be identified. All the metabolites that were identified by accurate mass, HRMS fragmentation spectrum and isotopic pattern match and/or match with authentic standards are reported in Table 1. More metabolites with their putative identification can be found in the Supporting Information Table S1.

Alcohol CMEs

The datasets of retention time, *m/z* pairs, and ion intensities were processed using PCA. As shown in the graph of Figure 2, time of exposure of the cannabis inflorescence to the solvent has a significant effect on small molecules, as reflected in the percentage of altered features in the alcohol CMEs (66% ESI+ and 79% in ESI− mode). The results showed a clear variation through three

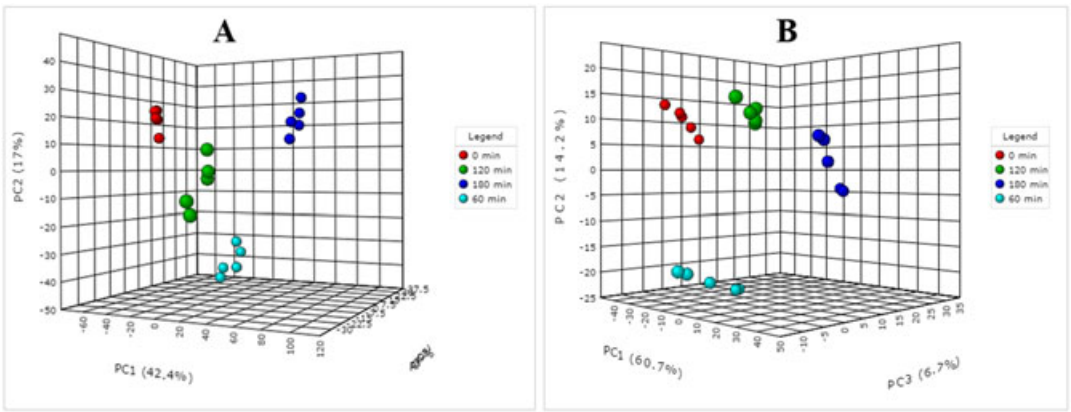


Figure 2. Three-dimensional principal component analysis (3D–PCA) of the extraction time points in ethyl alcohol in ESI+ and ESI− mode. (A) 3D–PCA of four datasets related to the four time points of extraction in ethyl alcohol acquired in ESI+ mode. (B) 3D–PCA of the same datasets acquired in ESI− mode. Samples extracted at t_0 are coloured in red, those extracted at 60 min in blue, those extracted at 120 min in green and those extracted at 180 min in dark blue. [Colour figure can be viewed at wileyonlinelibrary.com]

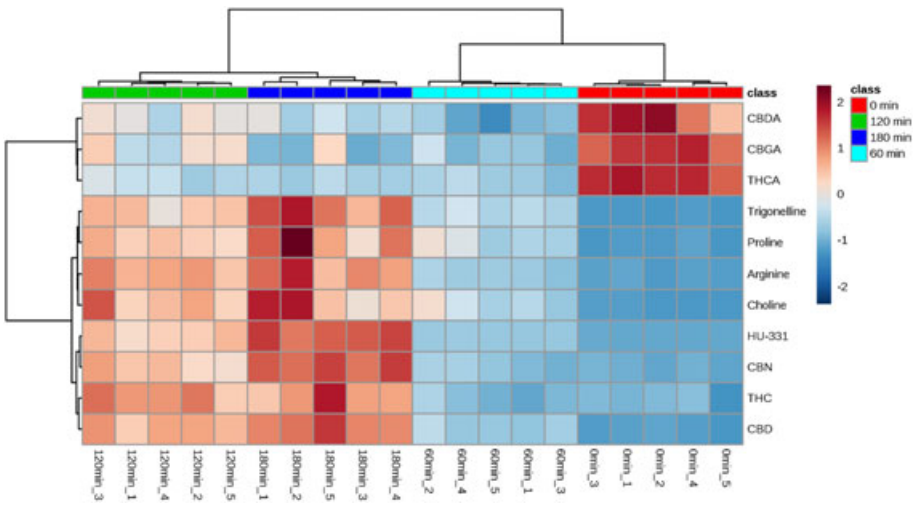


Figure 3. Heatmap of identified metabolites in ethyl alcohol in ESI+ mode. Colour-coding consists of shades of red and blue, where higher intensity of red means very high concentration and higher intensity of blue means very low concentration. The data of the selected time points are shown in colours at the top of the heatmap: t_0 is shown in red, 60 min in blue, 120 min in green and 180 min in dark blue. [Colour figure can be viewed at wileyonlinelibrary.com]

principal components (PC1, PC2 and PC3). The samples at 0, 60, 120 and 180 min formed separated clusters in the 3D–PCA scatter plot shown in Figure 2. The contribution of each principal component (PC) was 42.4% for PC1, 17% for PC2 and 8.6% for PC3 in ESI+ mode and 60.7% for PC1, 14.2% for PC2 and 6.7% for PC3 in ESI− mode. Multivariate analysis, performed with one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) Post-hoc analysis, revealed 3557 features that showed significant dysregulation among the four groups of extracts ($p < 0.01$) from 5446 total aligned features in ESI+ mode (Figure 2A). ESI− mode (Figure 2B) showed a higher percentage of altered features but a lower number of total aligned features (1617). Of these features in both ESI+ and ESI− mode, a number were adducts and fragment ions.

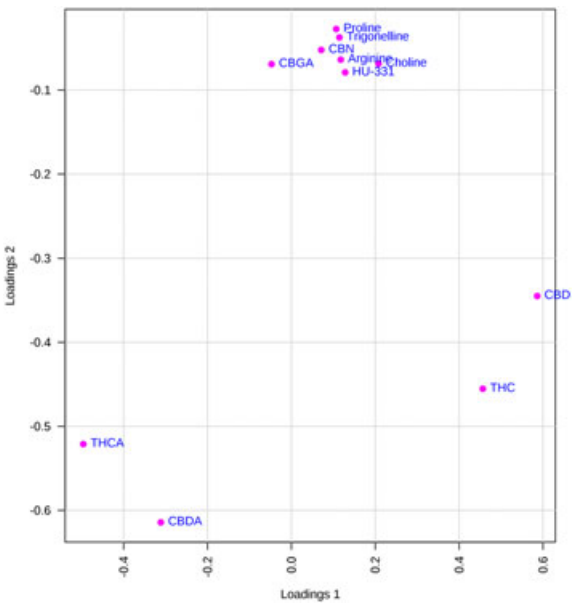


Figure 4. Principal component analysis (PCA) loadings of identified metabolites in ethyl alcohol cannabis medicinal extracts (CMEs) in ESI+ mode. Compounds with loadings far from zero (positive or negative) have a stronger contribution to creating the clusters separation. [Colour figure can be viewed at wileyonlinelibrary.com]

The results of the metabolomics experiments suggested that acid cannabinoids decarboxylate with different kinetics strictly dependent on both temperature and solvent. During the extraction process we observed an enrichment in acid cannabinoids that simultaneously decarboxylated and, in some cases, decomposed. The decomposition products often have an unknown structure, as well as their biological activity is unknown. The concentration of acid cannabinoids in alcohol CMEs showed a decrease over the time contributing to the clustering of the four groups of time points of extraction. The highest amount of CBGA, THCA and CBDA was registered at time t_0 since ethyl alcohol is a solvent with a high extraction efficiency and they began to be converted to their corresponding decarboxylated derivatives due to the temperature. Not much difference in THCA and CBDA concentrations was registered between 120 and 180 min. However, the concentration of neutral cannabinoids, such as CBD and THC, increased over the time as a result of the decarboxylation process of acid cannabinoids due to the temperature. In the last hour of extraction we observed very little increase of THC and CBD concentrations, suggesting that no more parent compound (THCA and CBDA) was being extracted. After 2 h of extraction, a small part of THC is also oxidised to CBN. Similarly, CBD underwent oxidation to give the quinone HU-331, which exerts antiangiogenic properties, induces apoptosis to endothelial cells and inhibits topoisomerase II in nanomolar concentrations (Peters and Kogan, 2007). The main acid and neutral cannabinoids, namely CBDA, CBGA, THCA, CBD, CBN and THC, were identified using authentic standards. The oxidation product of CBD, HU-331, was identified by accurate mass (Δppm <5), isotopic pattern and high-resolution fragmentation spectrum match with METLIN online database.

While the class of cannabinoids has been extensively studied, the composition of *C. sativa* L. in terms of primary and other secondary metabolites has not been completely elucidated. Compounds belonging to the primary metabolism are for example amino acids and fatty acids. We identified proline and arginine that showed an increasing trend over the time in ethyl alcohol. Other compounds, such as flavonoids, alkaloids, stilbenoids, terpenes, etc. are secondary metabolites. For some alkaloids like choline, which is one of the most important metabolites in plants since it is the precursor of the membrane phospholipid phosphatidylcholine (Rhodes and Hanson, 1993), an increase in concentration was

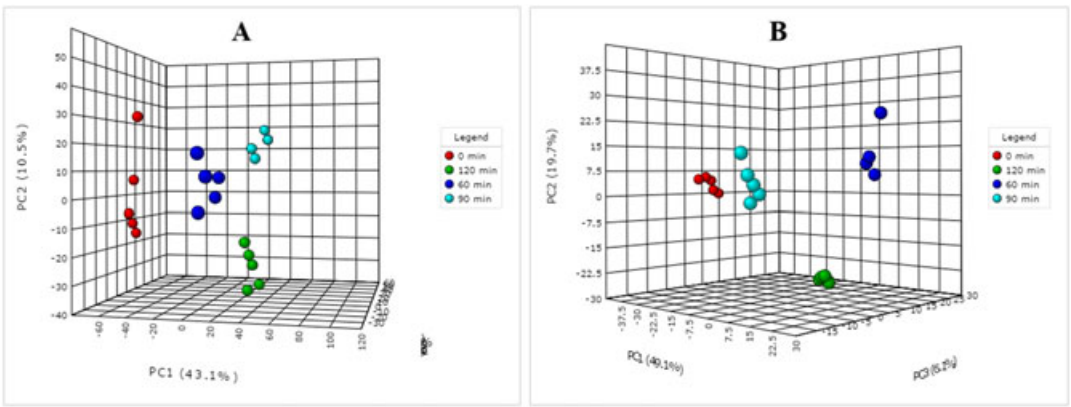


Figure 5. Three-dimensional principal component analysis (3D–PCA) of the extraction time points in olive oil in ESI+ and ESI− mode. (A) 3D–PCA of four datasets related to the four time points of extraction in olive oil acquired in ESI+ mode. (B) 3D–PCA of the same datasets acquired in ESI− mode. Samples extracted at t_0 are coloured in red, those extracted at 60 min in blue, those extracted at 120 min in green and those extracted at 180 min in dark blue. [Colour figure can be viewed at wileyonlinelibrary.com]

registered within the first 2 h; no much difference was observed after this time. The concentration of trigonelline, an alkaloid with pyridine structure and several pharmacological properties (Zhou *et al.,* 2012), increased with a regular trend over 3 h.

In order to have a clearer picture of the extraction trend of the identified metabolites, their raw data were used to build a heat map with MetaboAnalyst, where the individual values are represented as colours. The colour-coding is used to represent the values taken by a variable in a hierarchy (Figure 3). In the specific case only the metabolites identified in ESI+ mode were considered. Looking at the PCA loading plot in Figure 4 performed with the identified metabolites, the most discriminating features are the main acid and neutral cannabinoids, CBDA, THCA, CBD and THC.

Oil CMEs

Following the same procedure of alcohol CMEs, olive oil extracted samples were processed using PCA (Figure 5). Similarly, the four groups corresponding to the set time points clustered in different areas of the graph, suggesting a different chemical composition of the four groups of extracts. The contribution of each PC was 43.1% for PC1, 10.5% for PC2 and 6.9% for PC3 in ESI+ mode and 49.1% for PC1, 19.7% for PC2 and 6.1% for PC3 in ESI− mode. The number of the altered features detected in these extracts was lower than that of alcohol extracts; specifically, 3145 features out of 6193 were detected as altered (51%) in ESI+ mode (Figure 5A), whilst in ESI− mode (Figure 5B) the multivariate analysis identified 812 altered features out of 1181 (69%).

Ethyl alcohol and olive oil CMEs could be distinguished in terms of kinetics of extraction of both cannabinoids and secondary metabolites. In fact, whilst cannabinoids are more easily extracted by ethyl alcohol and more slowly in olive oil, secondary metabolites prefer more lipophilic solvents.

The results of the untargeted metabolomics studies on oil CMEs showed that acid cannabinoids (Figure 6) followed the opposite trend with respect to that observed in ethyl alcohol. This is most likely due to a different kinetics of extraction in olive oil that, being clearly slower than that in ethyl alcohol, produced an increase of their concentrations still after 2 h.

As for ethyl alcohol CMEs, a heatmap was generated with MetaboAnalyst to identify the extraction trend of the identified metabolites in olive oil. The heatmaps in Figures 3 and 6 highlight the differences between ethyl alcohol and olive oil with the colour coding in terms of extraction of the identified metabolites over the time in ESI+ mode. In particular, whilst carboxylated cannabinoids show their maximum intensity only at the beginning of the extraction process in ethyl alcohol to abruptly decrease over the first hour, in olive oil they are continuously extracted over 2 h. Decarboxylated cannabinoids levels increased over 3 h of extraction in ethyl alcohol, whereas they decreased after 90 min in olive oil. The maximum intensity of the metabolites peaks is also

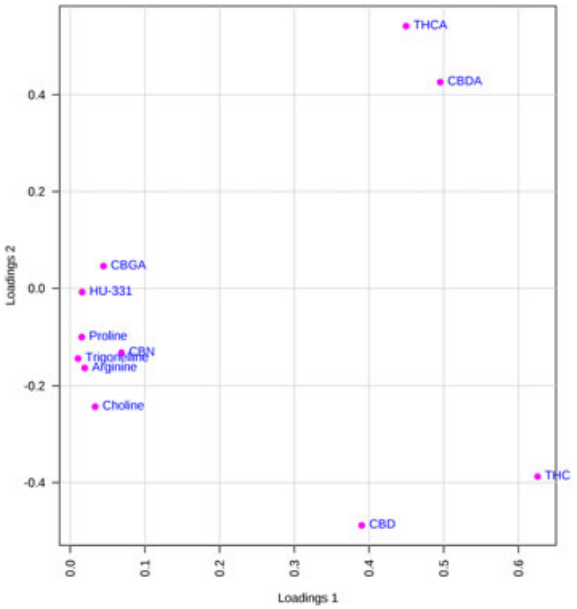


Figure 7. Principal component analysis (PCA) loadings of identified metabolites in olive oil cannabis medicinal extracts (CMEs) in ESI+ mode. Compounds with loadings far from zero (positive or negative) have a stronger contribution to creating the clusters separation. [Colour figure can be viewed at wileyonlinelibrary.com]

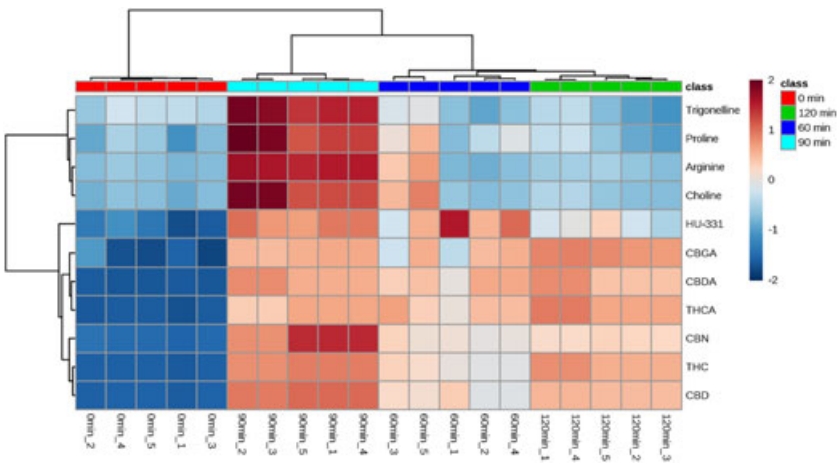


Figure 6. Heatmap of identified metabolites in olive oil in ESI+ mode. Colour-coding consists of shades of red and blue, where higher intensity of red means very high concentration and higher intensity of blue means very low concentration. The data of the selected time points are shown in colours at the top of the heatmap: t_0 is shown in red, 60 min in blue, 120 min in green and 180 min in dark blue. [Colour figure can be viewed at wileyonlinelibrary.com]

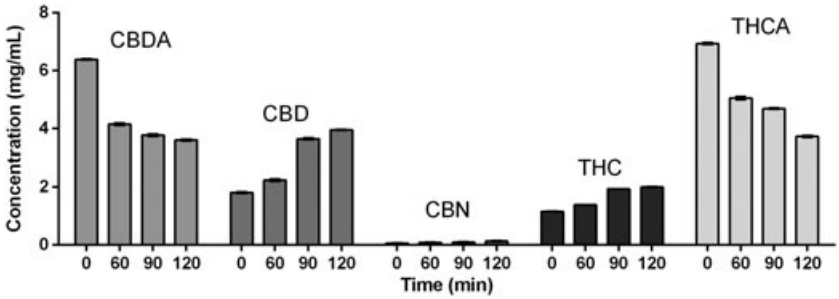


Figure 8. Cannabinoids concentration in ethyl alcohol at the different extraction time points. Cannabinoids concentrations (mg/mL, *y* axis) in ethyl alcohol acquired in ESI+ mode were plotted against time of extraction (min, *x* axis).

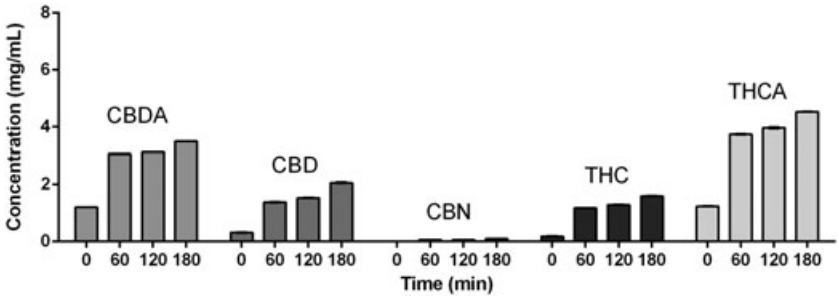


Figure 9. Cannabinoids concentration in olive oil at the different extraction time points. Cannabinoids concentrations (mg/mL, *y* axis) in olive oil acquired in ESI+ mode were plotted against time of extraction (min, *x* axis).

different between ethyl alcohol and olive oil: the maximum intensity, which is registered in ethyl alcohol at t_0, is not even reached in olive oil after 90 min, suggesting that ethyl alcohol possesses a higher extraction efficiency compared to olive oil. It is noteworthy that the contribution of the single metabolites to differentiate the chemical composition of four time points of CMEs is quite different in the two solvents.

The results of multivariate analysis suggested that cannabinoids and the ratio of acid to neutral cannabinoids are the most discriminating features in both olive oil and ethyl alcohol CMEs. As for alcohol CMEs, a PCA loading plot was generated with the identified metabolites. Figure 7 shows that, similarly to ethyl alcohol extraction, the main acid and neutral cannabinoids, CBDA, THCA, CBD and THC, have the strongest contribution to the clustering of the set time points.

Quantitative analysis of cannabinoids

Figures 8 and 9 illustrate the concentration of the main cannabinoids, namely CBDA, THCA, CBD, THC and CBN, at the selected time points of extraction in ethyl alcohol and olive oil, respectively. The concentrations were calculated with both UV and MS detection applying the analytical method recently published by our research group (Citti *et al.,* 2016a). Tables S2 and S3 (in Supporting Information) report the concentration values of the main cannabinoids for ethyl alcohol and olive oil, respectively. The results obtained with UV detector were consistent with those obtained with MS detector, confirming what has been previously published (Citti *et al.,* 2016a).

The results of the quantitative analysis indicated a significantly high extraction power of ethyl alcohol compared to olive oil. In particular, ethyl alcohol is able to extract 100% of the main acid cannabinoids in the early minutes of the contact with the cannabis inflorescence when the boiling point is reached. However, olive oil is not able to extract the acid cannabinoids as much as ethyl alcohol does probably due to its higher lipophilicity. Besides, during the process of decarboxylation the neutral cannabinoids are formed and solubilise into the solvent. These data suggested that the decarboxylation rate at 110°C of both CBDA and THCA is slower than the solubilisation rate. In the case of ethyl alcohol, instead, the decrease of the acid forms and the simultaneous increase of the neutral forms are exclusively due to the decarboxylation of the acid forms entirely dissolved in the early minutes of the extraction. The amount of total CBD is 7.11% (*w/w*) in alcohol CMEs and 5.13% (*w/w*) in oil CMEs. The amount of total THC is 5.35% (*w/w*) in alcohol CMEs and 5.57% (*w/w*) in oil CMEs.

The earlier mentioned provides a clear picture of the necessity of developing a standardised procedure for the extraction, especially in terms of time and solvent, since they unambiguously affect the chemical composition of the final CME, thus influencing the pharmacological effect of the drug that is eventually dispensed to the patients.

The complete characterisation of the chemical composition CMEs represents a substantive challenge. In this context, an untargeted metabolomic approach and a multi-group comparison of the different extracts revealed significant variations of the extracted metabolites concentrations. The data obtained suggested that the extraction procedure dramatically affects the chemical composition of the CMEs. When dealing with medicinal cannabis, which is intended for patients, it is important to know exactly the chemical content and the amount of each constituent of the final extract. A deeper investigation is certainly required to identify the hundreds, if not thousands, of molecules comprised in the cannabis phytocomplex. Indeed, our ongoing studies are devoted to the identification of a greater number of interesting and pharmacologically active molecules. The results presented herein

suggested that, notwithstanding the use of a standardised starting plant material, there is the urgent need to develop and standardise an extraction protocol in order to produce every time a known amount of cannabinoids and other bioactive molecules.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.