Analysis of impurities of cannabidiol from hemp. Isolation, characterization and synthesis of cannabidibutol, the novel cannabidiol butyl analog / Citti, C.; Linciano, P.; Forni, F.; Vandelli, M. A.; Gigli, G.; Lagana, A.; Cannazza, G.. - In: JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS. - ISSN 0731-7085. - 175:(2019), pp. 112752-112752. [10.1016/j.jpba.2019.06.049]

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PII:	S0731-7085(19)31013-1
DOI:	https://doi.org/10.1016/j.jpba.2019.06.049
Reference:	PBA 12752

To appear in:	*Journal of Pharmaceutical and Biomedical Analysis*

Received date:	24 April 2019
Revised date:	12 June 2019
Accepted date:	30 June 2019

Please cite this article as: Citti C, Linciano P, Forni F, Vandelli MA, Gigli G, Laganà A, Cannazza G, Analysis of impurities of cannabidiol from hemp. Isolation, characterization and synthesis of *cannabidibutol*, the novel cannabidiol butyl analog, *Journal of Pharmaceutical and Biomedical Analysis* (2019), https://doi.org/10.1016/j.jpba.2019.06.049

Analysis of impurities of cannabidiol from hemp. Isolation, characterization and synthesis of *cannabidibutol*, the novel cannabidiol butyl analog

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Highlights

- Cannabidivarin (CBDV) and cannabidibutol (CBDB) are the two major impurities in cannabidiol (CBD) extracted from hemp
- CBDB was isolated and fully characterized for the first time
- A stereoselective synthesis was carried out and absolute configuration assigned to natural CBD
- A perfect match of all properties of isolated CBDB and synthesized CBDB was obtained
- A simple and selective HPLC-UV method was developed and validated
- The method was applied to ten batches of commercial CBD marketed by certified companies

Abstract

Cannabidiol (CBD), one of the two major active principles present in *Cannabis sativa,* is gaining great interest among the scientific community for its pharmaceutical, nutraceutical and cosmetic applications. CBD can be prepared either by chemical synthesis or extraction from *Cannabis sativa* (hemp). The latter is more convenient from several points of view, including environmental and economic, but mainly for the absence of harmful organic solvents generally employed in the chemical synthesis. Although CBD produced by hemp extraction is the most widely employed, it carries two major impurities. The first one is the already known cannabidivarin (CBDV), whereas the second one is supposed to be the butyl analog of CBD with a four-term alkyl side chain. In this work, we report the isolation by semi-preparative liquid chromatography and the unambiguous identification of this second impurity. A comprehensive spectroscopic characterization, including NMR, UV, IR, circular dichroism and high-resolution mass spectrometry (HRMS), was carried out on this natural cannabinoid. In order to confirm its absolute configuration and chemical structure, the stereoisomer

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(1*R*,6*R*) of the supposed cannabinoid was synthesized and the physicochemical and spectroscopic properties, along with the stereochemistry, matched those of the natural isolated molecule. According to the International Nonproprietary Name, we suggested the name of *cannabidibutol* (CBDB) for this cannabinoid. Lastly, an HPLC-UV method was developed and validated for the qualitative and quantitative determination of CBDV and CBDB in samples of CBD extracted from hemp and produced according to Good Manufacturing Practices regulations for pharmaceutical and cosmetic use.

Keywords*: cannabidiol, cannabidivarin, cannabidibutol, CBD-*C_4*, LC-UV, LC-HRMS*

1. Introduction

Since its discovery by Adams in 1940 [1] and structure elucidation by Mechoulam and Shvo in 1963 [2], studies on cannabidiol (CBD, Figure 1) have undergone profound changes over the time. Initially, it was considered an inactive cannabinoid [3], thus leaving the field to the research on the "active" constituent of *Cannabis sativa*, Δ^9-tetrahydrocannabinol (Δ^9-THC). While deepening the knowledge on THC, the studies on CBD were confined to the interaction with the more interesting psychotropic isomer [3]. The period of silence on CBD eventually stopped in the early 2000's, when there was a boost in the number of publications on this cannabinoid due to the plethora of pharmacological activities addressed to CBD alone, many of which with therapeutic potential [3]. More than four hundred papers were published last year on CBD compared to about twenty exactly twenty years ago (from Scopus search "cannabidiol"). In 2018 CBD was approved by FDA for the treatment of severe forms of infant epilepsy (Lennox-Gastaut syndrome and Dravet syndrome) and it is now commercialized by GW Pharmaceuticals (UK) as a 100 mg/mL oral solution with the name of Epidiolex® [4]. CBD in Epidiolex is extracted from hemp inflorescence and therefore it is produced according to the Good Manufacturing Practices (GMP). GMP covers all stages of production from the starting materials to the facilities, equipment and processes, but also record keeping, personnel qualifications and training, sanitation and cleanliness. Hence, a drug substance that is produced as an Active Pharmaceutical Ingredient (API) should comply to a series of specifications including a detailed report of the chemical composition. In its application patent entitled "Use of cannabinoids in the treatment of epilepsy" [5], GW Pharmaceuticals lists the impurities in the extracted cannabidiol including cannabidiolic acid (CBDA) 0.15% w/w, cannabidivarin (CBDV, Figure 1) 1% w/w, Δ^9-THC 0.15% w/w and CBD-C_4 (Figure 1) 0.5% w/w. The latter is intended as 4-butyl-5'-methyl-2'-(prop-1-en-2-yl)-1',2',3',4'-tetrahydro-[1,1'-biphenyl]-2,6-diol, which is the analog of CBD with a butyl side chain in place of the conventional pentyl chain on the resorcinol moiety. However, no record of its physicochemical and optical characterization can be found in the scientific literature.

The same considerations on GMP are valid for other forms of CBD different from the oil, intended for pharmaceutical use, such as CBD crystals. CBD in this solid form can be obtained by either extraction from *Cannabis sativa* inflorescence or by a stereoselective synthesis. Natural CBD is generally extracted with organic solvents or by supercritical carbon dioxide from cannabis inflorescence, which has been previously decarboxylated since the plant only produces its acidic precursor CBDA [6]. Alternatively, CBDA can be extracted and then decarboxylated by heating the extract to get CBD [6, 7]. The extract usually needs to undergo a "winterization" or dewaxing step in order to remove the waxes and then CBD is purified. Purification can be performed by chromatography or directly crystallized from the winterized extract from either pentane or hexane [5, 8]. As an alternative, pure CBD can be produced via a stereoselective synthesis as reported by Petrzilka *et al.* [9] and later improved by Baek *et al.* [10]. The synthetic route involves the acid condensation of *p*-mentha-2,8-dien-1-ol with olivetol. However, beside CBD, as the major product, two main by-products are always obtained, namely a CBD isomer defined as “abnormal CBD” (*abn*-CBD, **1**, Figure 1) and a CBD with an additional *p*-mentha1,8-dien-3-yl moiety in 4’ position, namely 5,5''-dimethyl-6'-pentyl-2,2''-di(prop-1-en-2-yl)-1,1'',2,2'',3,3'',4'',6-octahydro-[1,1':3',1''-terphenyl]-2',4'-diol (**2**, Figure 1). Thus, chromatographic purification is required to obtained CBD with a degree of purity suitable for the pharmaceutical use, with consequent final yield not greater than 60%.

Therefore, from both economic and ecological point of view, the extraction of CBD from cannabis inflorescence still remains the most suitable process for industrial CBD production.

Notwithstanding the increasing use of CBD in pharmaceutical and cosmetic products, there is no related monograph in the official pharmacopoeias. The only official document reporting a protocol for solid or oily CBD formulations is a monograph in the German codex DAC/NRF, which has legal value only in Germany [11]. The monograph reports the main physicochemical properties, the methods for identification including thin layer chromatography, and the methods for determining the purity including liquid chromatography coupled to UV detection (HPLC-UV). The monograph also reports the impurities that can be encountered in a sample of solid CBD, specifically cannabinol (CBN), Δ^9-THC and Δ^8-THC, which together with other minor non specified impurities should be not more than 0.5% (w/w). The same monograph reports the preparation of an oily formulation of CBD 50 mg/mL in MCT (medium-chained triglycerides). However, the monograph does not mention two of the main impurities that can be found in CBD extracted from hemp like CBDV and CBD-C_4. The amount of the two impurities in the final product could be relatively high, up to 1% and 0.5% (w/w) for CBDV and CBD-C_4 respectively [5]. Although GMP procedures might be slightly different among countries, they all complies to general rules. Stacking to ICH guidelines, detection of impurities in an Active Pharmaceutical Ingredient (API) is regulated by the document Q3A(R2), which fixes the threshold for the determination of organic impurities according to the daily dose of the drug substance. Specifically, an impurity should be identified when present at a level of 0.10% and qualified at a level of 0.15% in a drug substance with a daily dose below 2 g/day. In a drug substance with a daily dose above 2 g/day, an impurity should be identified and qualified when present at a level of 0.05%. According to these guidelines, both impurities CBDV and CBD-C_4 should be qualified and reported in the certificate of analysis of the CBD product. To this end, suitable analytical methods should be applied to quantify these two compounds. A certified analytical standard for CBDV is commercially available and few analytical methods for its quantitative determination can be found in the literature [7, 12]. On the other hand, no analytical standard is available for CBD-C_4 and no analytical method has been published. Moreover, its identification in cannabis samples or CBD products has been obtained by only means of mass spectrometry data. No further characterization has been performed and the cannabinoid has never been isolated for confirmation. To the best of our knowledge and according to exact structure search on SciFinder, the most comprehensive database for chemical literature, only three scientific papers reports the mass spectrometry profile of CBD-C_4 using either gas chromatography analysis coupled to mass spectrometry (GC-MS) [13, 14] or Sorptive Tape-like Extraction coupled with Laser Desorption Ionization Mass Spectrometry (STELDI-MS) [15].

In the light of the above, the aim of the present work was to provide a full chemical characterization of CBD-C_4 including high-resolution mass spectrometry data (MS and MS/MS in positive and negative ionization mode) and spectroscopic data [NMR (^{1}H, ^{13}C, COSY, HSQC and HMBC), IR, UV, circular dichroism (CD) and optical rotatory power]. In order to confirm the identity of CBD-C_4, a stereoselective synthesis of the *trans* isomer (1*R*,6*R*) was carried out and all chemical properties were compared with those of CBD-C_4 directly isolated from commercial CBD crystal (extracted from hemp and produced according to GMP regulations) by semi-preparative liquid chromatography. Confident of the chemical identity of this cannabinoid, according to the International Nonproprietary Name (INN), we suggested for this CBD analog the name “cannabidibutol” (CBDB). With the pure analytical standard of CBDB in hand, a simple and sensitive liquid chromatography method coupled to UV detection (HPLC-UV) was developed and validated *ad hoc* in order to quantity both CBDV and CBDB in CBD samples extracted from hemp. The method was validated according to ICH guidelines (Q2(R1)) in terms of selectivity, linearity, accuracy, precision, dilution integrity and stability. Lastly, it was successfully applied to ten commercially available CBD samples marketed by certified companies.

2. Experimental

2.1 Chemicals and Reagents

Ethanol 96% analytical grade was bought from Carlo Erba (Milan, Italy). Acetonitrile, water and formic acid were all LC-MS grade and purchased from Carlo Erba. Cannabidivarin (CBDV) was purchased as a Cerilliant certified analytical standard (Sigma-Aldrich, Milan, Italy). Ibuprofen (Figure 1) was bought from Farmalabor (Canosa di Puglia, Italy). Samples of pure cannabidiol (extracted from hemp) were kindly provided by three companies: Ricerche Sperimentali Montale S.P.A. (Montale, Italy), Fagron Italia (Bologna, Italy) and CBDepot (Prague, Czech Republic). (1*S*,4*R*)-1-methyl-4-(prop-1-en-2-yl)cycloex-2-enol and 5-butylbenzene-1,3-diol were purchased from Combi-blocks (San Diego, CA, USA) and GreenPharma (Foligno, Italy), respectively. Chemicals and solvents necessary for the synthesis were reagent grade and used without further purification.

2.1 Synthesis of (1'*R*,2'*R*)-5'-methyl-4-pentyl-2'-(prop-1-en-2-yl)-1',2',3',4'-tetrahydro-[1,1'-biphenyl]-2,6-diol, (-)-*trans*-Cannabidibutol (CBDB)

To a solution of 5-butylbenzene-1,3-diol (83 mg, 0.50 mmol, 1 eq.) and *p*-toluenesulfonic acid (9 mg, 0.05 mmol, 0.1 eq.) in dry dichloromethane (DCM) (5 mL) at -10 °C, under argon atmosphere, a solution of (1*S*,4*R*)-1-methyl-4-(prop-1-en-2-yl)cycloex-2-enol and 5-butylbenzene-1,3-diol (76 mg, 0.50 mmol, 1 eq.) in 5 mL of dry DCM was added dropwise. The mixture was stirred in the same conditions for 1 h and then quenched with a saturated solution of $NaHCO_3$ (10 mL). The resulting mixture was extracted with diethyl ether (2×10 mL). The combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The crude was purified over silica gel (crude:silica gel ratio 1/200, eluent: cyclohexane:DCM 8/2) and all the chromatographic fractions were analyzed by HPLC-UV and UHPLC-HESI-Orbitrap. The fractions containing exclusively CBDB without impurities were collected to give 48 mg of a reddish oil (32% yield, purity >99%).

2.2 Isolation of natural cannabidibutol

A sample of commercial CBD crystals (1 g) was dissolved in acetonitrile (10 mL) and 0.5 mL aliquots of the solution were injected in a semi-preparative LC system (Octave 10 Semba Bioscience, Madison, USA). The separation was carried out on a fully porous silica stationary phase (Luna 5 µm C18(2) 100 Å, 250×10 mm) (Phenomenex, Bologna, Italy) with a mobile phase composed of acetronitrile:0.1% aqueous formic acid 70:30 (v/v) at a flow rate of 5 mL/min.

The impurity CBDV eluted at about 12 min, CBDB eluted at about 14 min and CBD eluted between 15 and 20 min. The fractions containing CBDV and CBDB were collected and analyzed by analytical HPLC-UV. The fractions containing exclusively CBDB were combined and dried on the rotavapor at 70 °C. An amount of about 1 mg of CBDB was obtained as a reddish oil.

2.3 Chemical and spectroscopic characterization of cannabidibutol

One-dimensional ^{1}H and ^{13}C NMR and two-dimensional NMR (COSY, HSQC and HMBC) were acquired on a DPX-600 Avance (Bruker) spectrometer (600.13 MHz for ^{1}H NMR and 150.92 MHz for ^{13}C NMR). A 10 mg aliquot of synthetic CBDB and 1 mg aliquot of CBDB isolated from CBD were solubilized in 700 and 250 µL of $CDCl_3$ (at 99.96% of deuteration) and placed in a 5 mm and 3 mm NMR tube, respectively. All NMR spectra were recorded at 298 K. ^{1}H-NMR were acquired with a spectral width of 13204.2 Hz, a relaxation delay of 5 s, a pulse width of 11.23 Hz and 16 number of transient. Proton chemical shifts were reported in parts per million (ppm, δ units) and referenced to the solvent residual peaks ($CDCl_3$ $\delta = 7.26$ ppm). Coupling constants are reported in Hertz (Hz). Splitting patterns are designed as s, singlet; d, doublet; t, triplet; q, quartet; dd, double doublet; m, multiplet; b, broad. ^{13}C-NMR were acquired with a spectral width of 33.3 kHz, a relaxation delay of 5 s, a pulse width of 10.00 Hz and 128 and 10240 number of transient for synthetic CBDB and extracted CBDB, respectively. Carbon chemical shifts were reported in parts per million (ppm, δ units) and referenced to the solvent residual peaks ($CDCl_3$ $\delta = 77.20$ ppm). The COSY were recorded as a 1024 × 160 matrix with 2 transients per t1 increment and processed as a 1024 × 1024 matrix. The HSQC spectra were collected as a 1024 × 256 matrix with 4 transients per t1 increment and processed as a 1024 × 1024 matrix, and the one-bond heteronuclear coupling value was set to 145 Hz. The HMBC spectra were collected as a 2048 × 220 matrix with 8 transients per t1 increment and processed as a 2048 × 1024 matrix, and the long-range coupling value was set to 8 Hz. IR spectra were recorded at 25 °C on a Perkin-Elmer Spectrum Two ATR-IR, scanning from 450 to 4000 cm^{-1}. Circular dichroism (CD) and UV spectra were acquired on a Jasco (Tokyo, Japan) J-1100 spectropolarimeter using a 50 nm/min scanning speed. Quartz cells with a 10 mm path length were employed to record spectra in the 400-200 nm range. Optical rotation (α) was measured with the P-2000 Digital Polarimeter (cell-length 100 mm, volume 1 mL) from Jasco Europe (Milan, Italy).

2.4 HPLC-UV analyses

High performance liquid chromatography (HPLC) analyses were carried out on an Agilent 1220 Infinity LC System (Waldbronn, Germany), consisting of a vacuum degasser, a binary pump, a manual injector, a column compartment and a UV detector. The separation of the analytes was performed with a Poroshell 120 C18 column (Poroshell 120 SB-C18, 3.0 × 150 mm, 2.7 µm, Agilent, Milan, Italy) eluting a mobile phase composed of 0.1% formic acid in both (A) water and (B) acetonitrile (ACN). An isocratic elution with 70% B was set for 10 minutes, then 95% B was pumped for 5 min and re-equilibration of the column was set for 2 min for a total run time of 17 min. The flow rate was maintained constant at 0.5 mL/min. The loading loop capacity was 6 µL. The loop was washed before each run first with 50 µL of ethanol 96% then with 50 µL of mobile phase. The UV trace was acquired at 228 nm. The analytes peaks were manually integrated using the EZChrom software (Agilent Technologies), which was employed also for controlling the online analysis.

2.5 UHPLC-HESI-Orbitrap mass spectrometry analyses

2.6 Ultra high-performance liquid chromatography (UHPLC) analyses were carried out for identification and purity test purposes. They were performed on a Thermo Fisher Scientific Ultimate 3000 equipped with a vacuum degasser, a binary pump, a thermostated autosampler, a thermostated column compartment and a Q-Exactive Orbitrap mass spectrometer with a heated electrospray ionization (HESI) source. The mass spectrometry parameters were optimized by direct infusion of the single analytes at the concentration of 1 µg/mL with a flow rate of 0.1 mL/min through a syringe pump. The HESI parameters were: capillary temperature, 320 °C; vaporizer temperature, 280 °C; electrospray voltage, 4.2 kV (positive mode) and 3.8 kV (negative mode); sheath gas, 55 arbitrary units; auxiliary gas, 30 arbitrary units; S lens RF level, 45. Control of online analyses was carried out using Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA). The exact masses of the compounds were calculated by the Qualbrowser in Xcalibur 3.0 software. The analyses were acquired in full scan data-dependent acquisition (FS-dd-MS^2) in positive and negative mode at a resolving power of 70,000 FWHM (full width at half maximum) at *m/z* 200. The other mass analyzer parameters were: scan range, *m/z* 250-400; AGC (automatic grain control) target, 3e6 ions in the Orbitrap analyzer; ion injection time, 100 ms; isolation window for the filtration of the precursor ions, *m/z* 2. Fragmentation of precursors was performed at 30 as normalized collision energy (NCE) by injecting working mix standard solution at a concentration of 5 µg/L. Detection was based on calculated [M+H]$^+$ and [M-H]$^-$ precursor ions with an accuracy of 2 ppm, retention time and fragmentation match (fragments *m/z* and intensity) with pure analytical standards. Analytical selectivity was assessed by UHPLC-HESI-Orbitrap MS using the same chromatographic conditions employed for the HPLC-UV method except for the different length of the column (Poroshell 120 SB-C18, 3.0 × 100 mm, 2.7 µm, Agilent, Milan, Italy).Preparation of standard solutions

A stock solution of internal standard (ibuprofen 10 mg/mL) was prepared by dissolving 100 mg in 10 mL of acetonitrile. Three serial 1/10 dilutions of the internal standard (IS) stock solution were performed to obtain 100 mL of IS working solution with the final concentration of 1 µg/mL in ACN. Stock solution of CBDV (1000 µg/mL in methanol) and CBDB (1000 µg/mL in ACN) were properly diluted in the IS working solution to obtain calibration standard solutions (CS) at the final concentrations of 0.28, 1.41, 2.82, 9.40, 28.2 and 56.4 µg/mL for CBDV and 0.12, 0.60, 1.20, 4.00, 12.0 and 24.0 µg/mL for CBDB. Independently prepared CBDV and CBDB mix solutions were prepared in IS and used as the low concentration quality control (LQC) (0.56 µg/mL for CBDV and 0.24 µg/mL for CBDB), medium concentration quality control (MQC) (18.8 µg/mL for CBDV and

8.00 µg/mL for CBDB), and high concentration quality control (HQC) (45.1 µg/mL for CBDV and 19.2 µg/mL for CBDB) samples. QCs were prepared as for calibration standards.

2.7 Method validation

In order to demonstrate the reliability and robustness of the method, a method validation was carried out based on EMA guidelines and in agreement with international guidelines for analytical techniques for the quality control of pharmaceuticals (ICH guidelines) [16, 17]. The method was validated in terms of selectivity, linearity, accuracy, precision, dilution integrity and stability. No matrix effect or recovery were assessed as the matrix is represented by acetonitrile, for which no matrix effect should be encountered as it is present in the mobile phase.

Selectivity. Selectivity is performed in order to assess the ability of the method to differentiate and quantify the analytes in the presence of other components in the sample. It was investigated by analyzing blank samples, samples containing the analytes and authentic standards and comparing the retention times of potential interfering compounds with those of reference standards and IS. Identity of the analytes was assessed by comparing accurate (within 2 ppm error) *m/z* of $[M+H]^+$ and $[M-H]^-$ ions and MS/MS spectra of analytical standards with those obtained by UHPLC-HESI-Orbitrap for the analytes in authentic samples.

Linearity. Calibration curve was constructed at six non-zero calibration levels 0.28, 1.41, 2.82, 9.40, 28.2 and 56.4 µg/mL for CBDV, 0.12, 0.60, 1.20, 4.00, 12.0 and 24.0 µg/mL for CBDB, and 1.00 µg/mL for IS. Peak area ratios of analyte-to-IS were plotted *vs* actual concentrations. Calibration curve was built at the beginning of each validation day of five consecutive days (n = 5). A linear correlation was assumed if the coefficient of determination (R^2) was greater than 0.998 using weighed regression method ($1/x^2$). The back calculated concentrations should be within 15% of the nominal concentrations, and within 20% of the lower limit of quantification (LLOQ).

Limit of detection (LOD) and limit of quantification (LOQ). Limit of detection (LOD) was estimated based on a 3:1 signal-to-noise (S/N) ratio. Standard stock solutions of the analytes were appropriately diluted at the levels of their respective estimated LOD values. The LOD values were then calculated as three times the standard deviation (SD) obtained by repeatedly analyzed standards (n = 5). Lower limit of quantification (LLOQ) was estimated based on a 10:1 S/N ratio and calculated as ten times the SD of repeatedly analyzed standards. The upper limit of quantification (ULOQ) was set at 10% above the highest concentration of the analytes in a concentrated sample of CBD (10 mg/mL).

Autosampler carryover. Autosampler carryover was evaluated by running two blank samples after a calibration standard at the ULOQ and after a high concentration sample (CBD 10 mg/mL). The carryover should not be greater than 20% of the LOQ for the analytes and 5% for IS.

Accuracy and precision. The precision and accuracy were evaluated at four levels, LLOQ (0.28 µg/mL for CBDV and 0.12 µg/mL for CBDB), LQC (0.56 µg/mL for CBDV and 0.24 µg/mL for CBDB), MQC (18.8 µg/mL for CBDV and 8.00 µg/mL for CBDB), and HQC (45.1 µg/mL for CBDV and 19.2 µg/mL for CBDB). Each sample was analyzed in triplicate within a single day to determine the intra-day precision and accuracy. The replicate analyses were repeated on freshly prepared standard solutions for five successive days (n = 15) to determine the inter-day precision and accuracy.

The precision was expressed as coefficient of variation (CV), and the accuracy was expressed as the percentage of mean calculated compared to nominal concentration.

Dilution integrity. Dilution integrity was carried out using a spiking standard solution of the analytes prepared by diluting standard stock solutions to a final concentration that is three times that of the ULOQ (170.4 µg/mL for CBDV and 72.00 µg/mL for CBDB). Dilution integrity was demonstrated by diluting the spiking solution in IS to 1/5, 1/10 and 1/20 of its original concentration. Five replicates per dilution factor were run. The concentrations were calculated by applying the dilution factor 5, 10 and 20 against freshly prepared calibration curve. Dilution integrity is ensured as long as precision and accuracy are ≤15% and ±15% respectively.

Stability. The short-term stability of the standard analytes was determined for LQC and HQC samples for 24 h at room temperature and under refrigeration (2-8 °C). The drugs were considered stable if the mean concentration (n = 3 for each sample) was within ±15% of the nominal concentration.

2.8 Preparation and analysis of CBD samples

Authentic CBD samples (API grade) were prepared by weighing 10 mg of solid crystals and dissolving them in 1 mL of IS working solution. A 100 µL aliquot of the solution was diluted with 900 µL of IS working solution to get a CBD concentration of 1 mg/mL. The HPLC-UV analyses of authentic CBD samples were carried out in triplicate employing the validated method described above.

3. Results and Discussion

3.1 Identification of CBD impurities by mass spectrometry

CBD samples were analyzed by UHPLC-HESI-Orbitrap, which represents an increasingly popular technology in the mass spectrometry field due to its high accuracy and precision (below 2 ppm error) in the determination of the exact mass and fragmentation profiles of organic compounds. The main peak in the chromatograms (Figure 2) was identified as CBD by overlap of the extracted ion chromatogram (EIC, Figure 3) and MS/MS spectra of the pure analytical standard of CBD analyzed in the same LC-MS conditions in both ESI+ and ESI- mode (Figure 4). The MS spectrum of the peak at 4.2 min does not show any other interfering compound. The precursor ion [M+H]+ has *m/z* 315.2314 and the [M-H]- has *m/z* 313.2179.

As shown in Figure 3, the peak at 2.6 min (impurity 1) corresponds to the ions [M+H]+ with *m/z* 287.2002 and [M-H]- with *m/z* 285.1861. Both MS/MS spectra (ESI+ and ESI-) in Figure 5 present a perfect match with those of cannabidivarin (CBDV) analyzed in the same conditions. The peak corresponding to this analyte does not present any interfering peak.

The peak at 3.3 min in Figure 3 (impurity 2) corresponds to the ions [M+H]+ with *m/z* 301.2157 and [M-H]- with *m/z* 299.2016. No interfering peak was detected at the retention time of this compound. Both MS/MS spectra (ESI+ and ESI-) of impurity 2 are shown in Figure 6. The elution time is between that of CBDV and CBD, indicating that the polarity of the compound is greater than CBD and lower than CBDV. The molecular ions [M+H]+ at *m/z* 301.2157 and [M-H]- at *m/z* 299.2016 correspond exactly to one methylene unit (-CH_2-) either inserted onto CBDV or removed from CBD.

The fragmentation pattern in both ESI+ and ESI- mode corroborates the hypothesis that the difference lies in a methylene unit since both fragments and ion abundance perfectly match with those of CBDV and CBD. As highlighted in Figure 7, which represents the fragmentation pattern of the three cannabinoids in positive ionization mode, the fragment at *m/z* 245.1533 in the MS/MS spectrum of impurity 2 has the same ionic abundance of the fragments at *m/z* 259.1960 and *m/z* 153.0909 in the MS/MS spectra of CBD and CBDV respectively. These fragments derive from the loss of four carbon units from the terpene moiety. Similarly, the fragment at *m/z* 221.1533 for impurity 2 derives from the breakage of the terpene group with only four carbon units left, as it also occurs for the fragments at *m/z* 235.1690 for CBD and *m/z* 207.1378 for CBDV. The base peak at *m/z* 179.1064 also differs by one methylene unit from the base peaks at *m/z* 193.1222 for CBD and *m/z* 165.0910 for CBDV, and corresponds to the complete loss of the terpene moiety except for one carbon unit. The fragment at *m/z* 167.1065 derives from the further loss of the last carbon unit of the terpene moiety to give the protonated molecule of resorcinol with four carbon atoms on the alkyl side chain. The corresponding fragments for CBD and CBDV are at *m/z* 181.1221 and *m/z* 153.0909, respectively. The other smaller fragments are enclosed in the blue dashed box and correspond to the fragmentation profile of the terpene moiety, which remains unchanged for all three cannabinoids. In a similar way, the fragmentation pattern of impurity 2 in negative ionization mode, shown in Figure 8, is identical to those of CBD and CBDV differing by a methylene unit. The base peak at *m/z* 231.1387 in the spectrum of impurity 2 derives from a retro Diels-Alder reaction and loss of a part of the terpene moiety, similarly to the fragments at *m/z* 245.1546 and *m/z* 217.1229 for CBD and CBDV respectively. The other important fragment is at *m/z* 165.0912 deriving from the complete loss of the terpene moiety that leads to the ionized resorcinol molecule. The corresponding fragments for CBD and CBDV are at *m/z* 179.1069 and *m/z* 151.0754 respectively. All fragments and molecular ions show the same ionic abundance. These data are in agreement with the hypothesis that impurity 2 is a cannabinoid with molecular formula $C_{20}H_{28}O_2$. The data are also in accordance with the high-resolution mass spectrometry characterization of CBD, CBDB and CBDV reported in the literature [18].

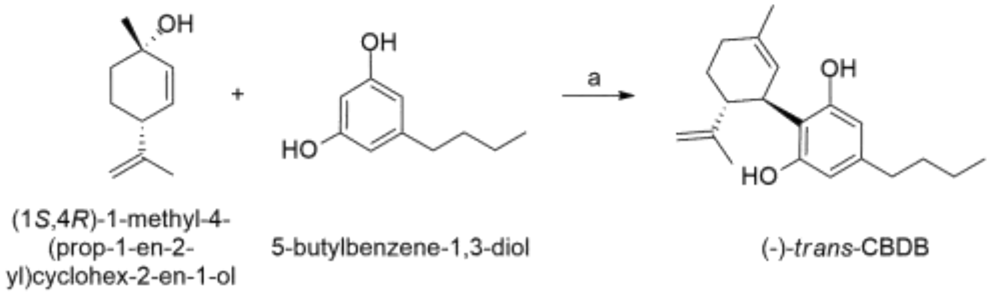
3.2 Isolation of natural CBDB

The isolation of impurity 2 from CBD is necessary in order to give an unambiguous identification. To this end, a semi-preparative chromatographic method was developed employing a semi-preparative column with a fully porous C18 silica stationary phase (250 × 10 mm) and a mobile phase composed of ACN and water 70:30 (v/v). This method allowed for the isolation of about 1 mg of impurity 2 with a purity greater than 99% starting from 1 g of a commercial hemp derived CBD. The amount of isolated compound resulted sufficient to perform a comprehensive spectroscopic characterization, including NMR, optical rotatory power, UV and CD (Supporting Information), in order to confirm its chemical structure by comparison with the spectroscopic data of synthetic CBDB.

3.3 Synthesis of CBDB

To best of our knowledge, the synthesis and full spectroscopy characterization of CBDB has never been reported in the literature and its existence has been hypothesized only by means of MS data. In absence of an analytical standard or any spectroscopic reference of CBDB that could be used for the identification of impurity 2, we needed to synthesize and carry out a full spectroscopic profile of the molecule (-)-*trans*-CBDB. The latter was prepared by Friedel-Craft allylation of 5-butylbenzene-1,3-

diol with (1*S*,4*R*)-1-methyl-4-(prop-1-en-2-yl)cycloex-2-enol, using *p*TSA as catalyst as reported in Scheme 1.



Scheme 1. *Reagents and conditions: a) pTSA (0.1 eq.), DCM dry, Argon, -10 °C, 1h, 32% yield.*

3.4 Spectroscopic characterization of *syn*-CBDB and *ext*-CBDB

The chemical identity of synthetic CBDB and its unambiguous ^{1}H assignments were achieved by analysing ^{1}H-NMR, ^{13}C-NMR and COSY spectra. The protonated carbon atoms were assigned by analysis of the HSQC spectra, and the quaternary carbons were assigned based on HMBC spectra. Table 1 and Figure 9 show the ^{1}H and ^{13}C NMR complete assignments for CBDB, confirming the chemical structure of the new synthesized cannabinoid. However, since the molecule of CBDB possesses two stereocenters, it could exist as four possible stereoisomers. Although confident that the synthetic procedure adopted leads to the stereoselective synthesis of (-)-*trans*-CBDB, the exact position of the double bonds and the configuration of carbon 1 and 6 were fully investigated and determined by NMR.

Indeed, even though the NMR is not able to discriminate between a couple of enantiomers, a slight shift of the proton signals could be detected among a couple of diastereomers (i.e. cis/trans isomers). Because CBDB and CBD differ only for a methylene in the alkyl chain on the resorcinol moiety, no significative difference in the proton chemical shifts of the terpene moiety are expected among the two molecules. Thus, in order to define first a *cis* or *trans* configuration for the synthetic CBDB, we compared its ^{1}H-NMR with the spectra of (-)-*trans*-CBD [19] and (-)-*cis*-CBD [9]. As reported in Table 2, it is possible to observe a close match between the H-1, H-2 and H-6 signals of synthetic CBDB and the corresponding signals of (-)-*trans*-CBD. In contrast, *cis*-CBD presents a shielding for H-1 and H-2 (from 3.81-3.84 to 3.35-3.75 and from 5.54 to 5.40, respectively) and a deshielding for H-6 (from 2.38 to 2.90). These outcomes suggested, therefore, a *trans* configuration for the synthetic CBDB. However, as *trans*-CBDB can exist as an enantiomeric couple (1*R*,6*R*) or (1*S*,6*S*), in order to establish the absolute configuration of C-1 and C-6, the optical rotatory power was determined, resulting in an $[\alpha]_{D}^{20} = -121°$ (*c.* 1.6, ACN). This value is in line with the $[\alpha]_{D}^{20} = -125°$ of (-)-*trans*-CBD, allowing the assignment of the (*1R,6R*) absolute configuration and the unambiguous identification of the synthetic CBDB as (-)-(*1R,6R*)-CBDB.

Confident of the chemical structure and stereochemistry of the synthesized (-)-*trans*-CBDB, the latter was used as reference compound to confirm the identity of the impurity 2. The NMR spectra, UPLC retention time, *m/z* precursor ions ($[M+H]^{+}$ and $[M-H]^{-}$), MS, UV and CD spectra of both synthetic CBDB and impurity 2 (isolated natural CBDB) were compared. As reported in figure 10, a perfect superimposition between the spectroscopic data of the two molecules was observed. Moreover, the specific rotatory power of isolated CBDB resulted in an $[\alpha]_D^{20}$ = -116° (*c.* 0.5, ACN) comparable to the value obtained for the synthetic CBDB.

Therefore, based on these considerations, we can state that the impurity 2 present in the CBD extracted from hemp inflorescence is (-)-*trans*-CBDB.

3.4 HPLC-UV method development and validation

Our research group has recently published several works on the development and optimization of analytical methods for the separation of cannabinoids by LC-UV and LC-MS [7, 12, 18, 20-23]. In order to achieve the optimal resolution of the analytes CBDV and CBDB, a core shell column (Poroshell 120 SB-C18, 3.0 × 150 mm, 2.7 µm) was employed. The mobile phase consisted of 0.1% aqueous formic acid and acetonitrile (30:70, v/v), which allowed for the separation of the analytes within 7 min and of the main peak of CBD within 10 min. Figure S2 in the Supplementary material shows the LC-UV chromatograms of a standard mixture at different concentration levels and an example of an authentic CBD sample. In order to assess the reliability and robustness of the method, a validation according to EMA and ICH guidelines was performed in terms of linearity, selectivity, carryover, accuracy, precision, dilution integrity and stability [16, 17], and the results are described below. The tables of the validation results are reported in the Supplementary material.

Linearity. Linearity was assessed for CBDV and CBDB in the ranges 0.28-56.4 µg/mL for CBDV and 0.12-24.0 µg/mL for CBDB. The concentration-response relationship was based on a weighted linear regression ($1/x^2$) in order compensate for the error at low concentrations considering the high dynamic range covered by the calibration curve. The coefficient of determination (R^2) was found above 0.999 for both analytes CBDV and CBDB, thus the correlation of concentration *vs* UV response was considered linear in the specified range (Table S1).

Limit of quantification (LOQ) and limit of detection (LOD). LOD was calculated as described in the methods section and was found 0.10 µg/mL for CBDV and 0.04 µg/mL for CBDB. The LLOQ, which is also the lowest calibration point was 0.28 µg/mL for CBDV and 0.12 µg/mL for CBDB. The ULOQ was set 10% above the highest concentration found for the analytes by injecting a high concentration CBD sample (10 mg/mL). Considering that the highest concentration of CBDV and CBDB in that concentrated CBD sample was 51.0 and 21.0 µg/mL, respectively, the ULOQ was set at 56.4 and 24.0 µg/mL for CBDV and CBDB, respectively.

Autosampler carryover. The analyses performed to assess the autosampler carryover indicated that the peak area corresponding to the analytes was not greater than 17% of the LLOQ and it was totally absent for the IS, thus ensuring good reliability of the quantification of the analytes.

Accuracy and precision (CV). Intra-day accuracy ranged from 98.23 to 104.9% for CBDV and from 100.3 to 105.5% for CBDB. Intra-day precision, expressed as coefficient of variation (CV), was found in the range 0.98-2.25% for CBDV and in the range 1.62-12.0% for CBDB. Inter-day accuracy ranged from 102.0 to 109.0% for CBDV and from 91.67 to 102.0% for CBDB. Inter-day CV was in the range 0.96-2.76% for CBDV and in the range 2.37-9.14% for CBDB. The acceptance criteria of EMA guidelines are established in the range 85-115% for accuracy and below 15% for CV (Table S2). Given the data above, it can be inferred that the developed method is accurate and precise.

Dilution integrity. For dilution integrity accuracy and CV were evaluated across five analyses of a highly concentrated standard mixture of CBDV and CBDB prepared with a concentration three times higher than the ULOQ and diluted to 1/5, 1/10 and 1/20. The accuracies were found in the range 96.72-99.53% for CBDV and in the range 93.47-96.00% for CBDB. The CV was below 3% for both analytes (Table S3). Since accuracy and precision were within ±15% of the nominal concentration, the results met the EMA acceptance criteria, ensuring that samples with concentrations greater than the ULOQ could be diluted and quantified with a good level of confidence. Moreover, this suggests that the calibration range could be extended above the ULOQ set in this method.

Stability. The stability of the analytes was evaluated at two concentration levels, LQC and HQC, at two different temperatures, room temperature (25 °C) and under refrigeration (2-8 °C), in a time interval of 24 hours. The analytes were found stable in both conditions as the calculated concentration was within 5% of the nominal concentration using a freshly prepared calibration curve (Table S4).

3.5 Analyses of authentic CBD samples

Authentic CBD samples (1 mg/mL), coming from different batches produced according to GMP regulations by hemp extraction and provided by three manufacturers, were analyzed according to the developed HPLC-UV method as described in the experimental section. The results obtained for the concentrations of CBDV and CBDB present in the samples are reported in Table 3. These impurities were present in amounts lower than 0.5%, in particular CBDV was found in the range 0.07-0.41% and CBDB was in the range 0.08-0.19%. The values are extremely variable across the ten samples, most likely because both hemp variety and industrial product manufacturing affect the amount of impurities eventually present in the final product. CBDV has already been detected in several hemp varieties in extremely variable concentrations [7, 24-27]. CBDB was also detected in some hemp varieties but its concentrations have never been determined due to the lack of the corresponding analytical standard [13, 14, 28]. It is reasonable to assume that the concentrations of CBDV and CBDB in CBD samples are a mirror of the concentrations of these analytes in the original plant material from which CBD is extracted. Moreover, since CBD is generally extracted from hemp by crystallization without further purification, the structural similarity of CBDV, CBDB and CBD leads to a co-crystallization of the three compounds. Chromatography would be the only means that can allow to remove such impurities and obtain a 99.99% pure CBD. However, this involves the use of organic solvents, which in turn can be found as a residual impurity in the final product, thus representing a detrimental method from an ecological point of view.

4. Conclusions

One of the major impurities of CBD extracted from hemp, cannabidibutol (CBDB), was fully characterized for the first time. A stereoselective synthesis was developed in order to confirm its identity and stereochemistry. This allowed to obtain for the first time the authentic analytical standard, which was employed for the development and validation of an HPLC-UV method for its qualitative and quantitative determination in commercial samples of CBD produced according to GMP regulations. Such standard and analytical method will bridge the gap of an essential need of pharmaceutical and cosmetic industries that produce CBD. Moreover, although the monograph of CBD in the German DAC code does not mention the presence of either CBDV or CBDB, the latter are undoubtedly the two major impurities in CBD extracted and crystallized from hemp. In our opinion, these two impurities should be included along with the analytical method for their determination in a desirable monograph on CBD of an official pharmacopoeia. Considering that CBDB is present in hemp, as reported by few articles, it can most likely be found also in the acidic form as cannabidibutolic acid (CBDBA), without ruling out the presence of the corresponding ring-closed isomer, tetrahydrocannabutolic acid (THCBA), and of the neutral derivative tetrahydrocannabutol (THCB). The ongoing research in our laboratory aims at identifying these molecules in different cannabis varieties.

Acknowledgements

This work was supported by UNIHEMP research project "Use of iNdustrIal Hemp biomass for Energy and new biocheMicals Production" (ARS01_00668) funded by Fondo Europeo di Sviluppo Regionale (FESR) (within the PON R&I 2017-2020 – Axis 2 – Action II – OS 1.b). Grant decree UNIHEMP prot. n. 2016 of 27/07/2018; CUP B76C18000520005 – COR 571294.
Also, this research work has been partly funded by the project "Development of a cannabis based galenical preparation" FONDO DI ATENEO PER LA RICERCA ANNO 2017 - FAR2017, Italy (A.006@FAR2017DIP@05FA-CANNAZZA_FAR2017-(.20) CUP E53C17000720005).

The authors would like to thank Boris Bañas of CBDepot (Prague, Czech Republic), RSM S.p.a. (Montale, Italy), Linnea SA (Riazzino, Switzerland), Crystal Hemp (Lugano, Switzerland), Farmacia Sant'Elia (Corato, Italy) and Farmacia Tundo Dr. Alfredo (Alliste, Italy) for providing authentic CBD samples.

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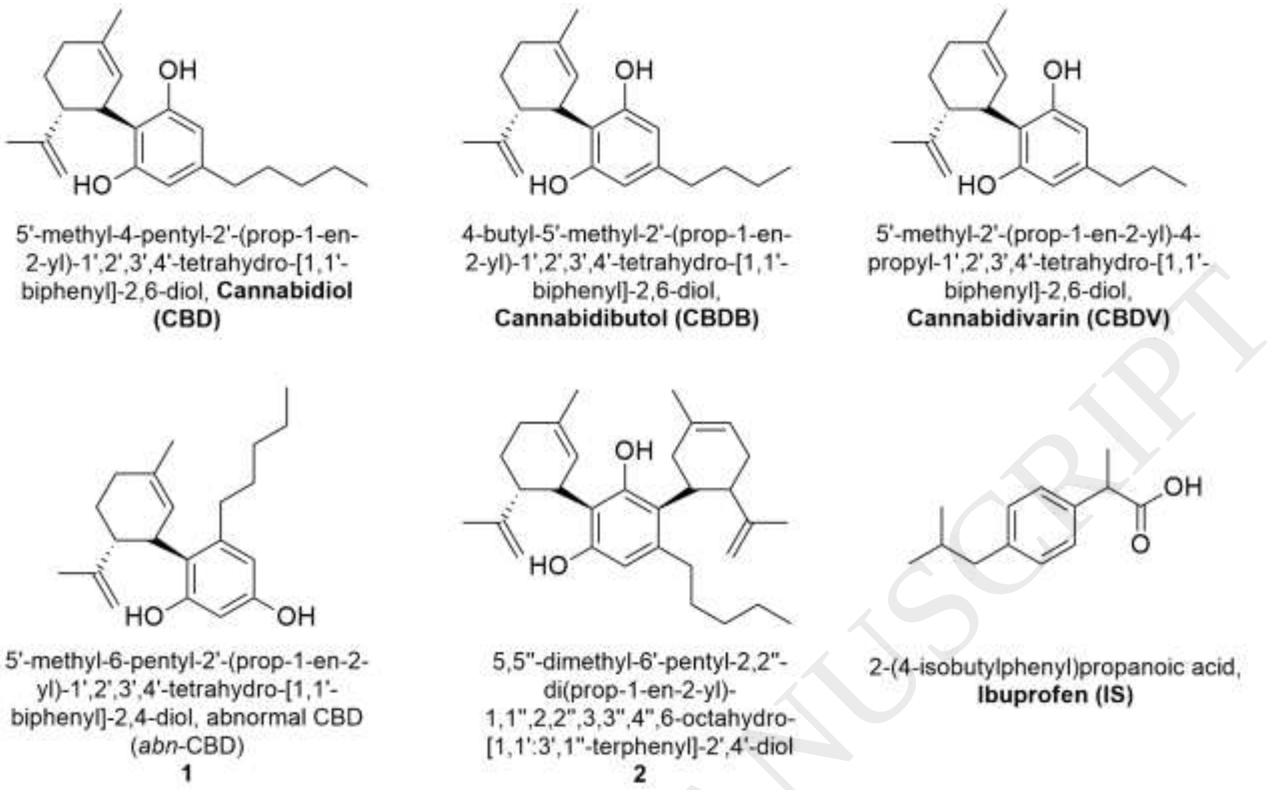
Legend to figures



5'-methyl-4-pentyl-2'-(prop-1-en-2-yl)-1',2',3',4'-tetrahydro-[1,1'-biphenyl]-2,6-diol, **Cannabidiol (CBD)**

4-butyl-5'-methyl-2'-(prop-1-en-2-yl)-1',2',3',4'-tetrahydro-[1,1'-biphenyl]-2,6-diol, **Cannabidibutol (CBDB)**

5'-methyl-2'-(prop-1-en-2-yl)-4-propyl-1',2',3',4'-tetrahydro-[1,1'-biphenyl]-2,6-diol, **Cannabidivarin (CBDV)**

5'-methyl-6-pentyl-2'-(prop-1-en-2-yl)-1',2',3',4'-tetrahydro-[1,1'-biphenyl]-2,4-diol, abnormal CBD (*abn*-CBD) **1**

5,5"-dimethyl-6'-pentyl-2,2"-di(prop-1-en-2-yl)-1,1",2,2",3,3",4",6-octahydro-[1,1':3',1"-terphenyl]-2',4'-diol **2**

2-(4-isobutylphenyl)propanoic acid, **Ibuprofen (IS)**

Figure 1. Structure and names (IUPAC, INN and abbreviation) of cannabidiol (CBD), cannabidibutol (CBDB), cannabidivarin (CBDV), main by-products of synthesis of CBD (**1** and **2**) and ibuprofen (used as internal standard for HPLC-UV method).

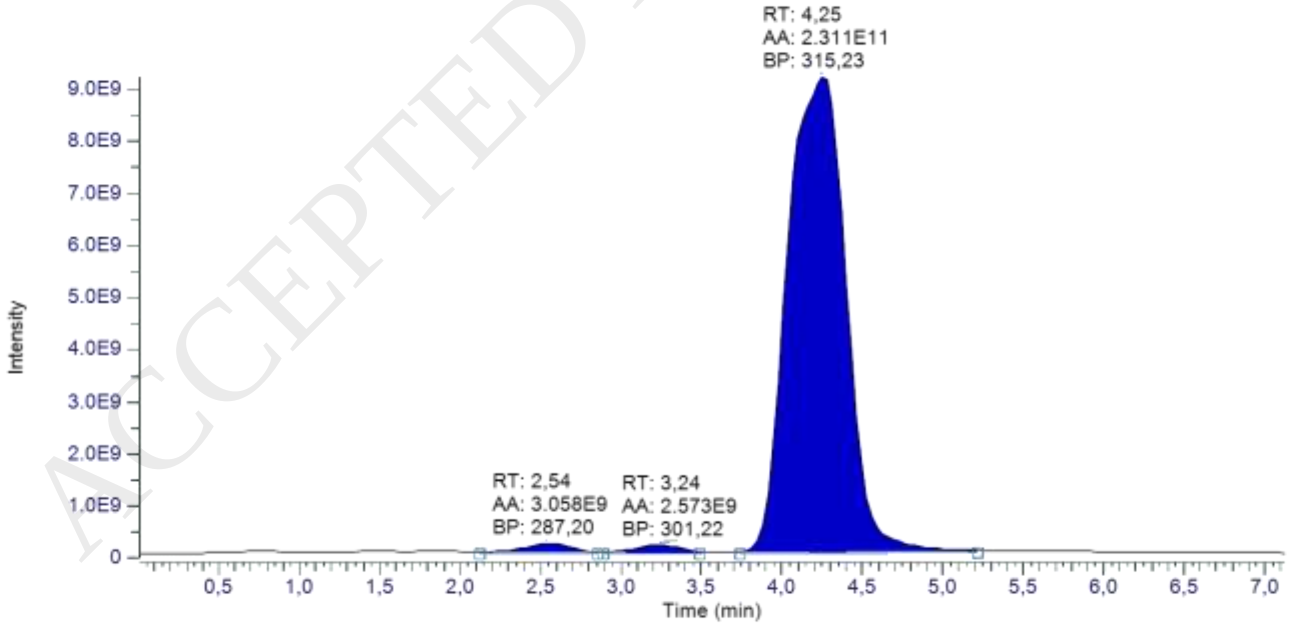


Figure 2. Total Ion Chromatogram (TIC) of a CBD authentic sample. The mail peak is identified as CBD, the other two peaks at 2.54 and 3.24 min are impurity 1 and impurity 2 respectively.

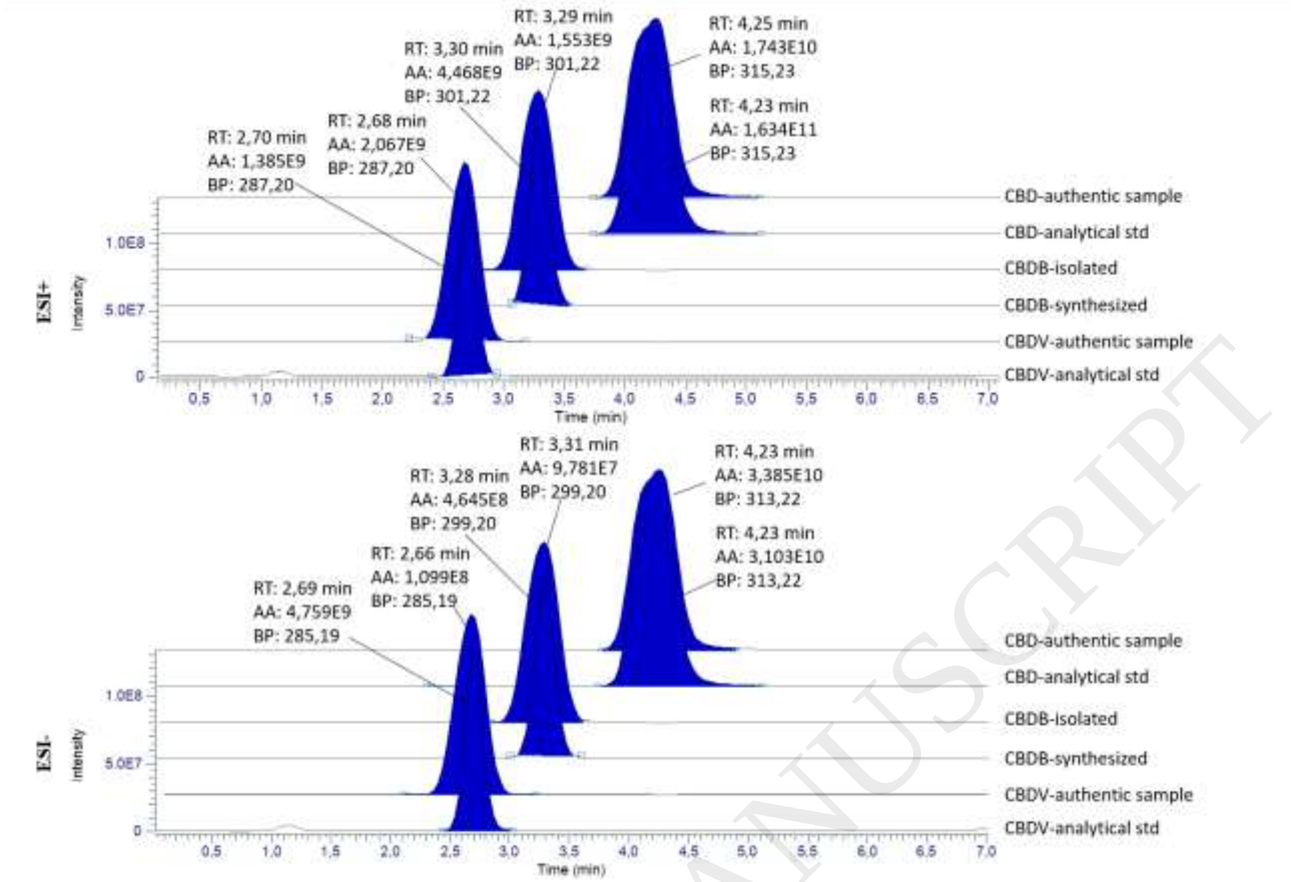


Figure 3. Extracted Ion Chromatograms (EIC) of CBD, CBDB and CBDV in positive (top) and negative (bottom) ionization mode. Area of the peaks were obtained by extracting the exact mass (Δppm=2) of CBD ($[M+H]^+$ 315.2314, $[M-H]^-$ 313.2179), CBDB ($[M+H]^+$ 301.2157, $[M-H]^-$ 299.2016) and CBDV ($[M+H]^+$ 287.2002, $[M-H]^-$ 285.1861). The chromatograms show the exact match of CBD and CBDV in samples with authentic analytical standards and the match of synthetic CBDB with natural isolated CBDB.

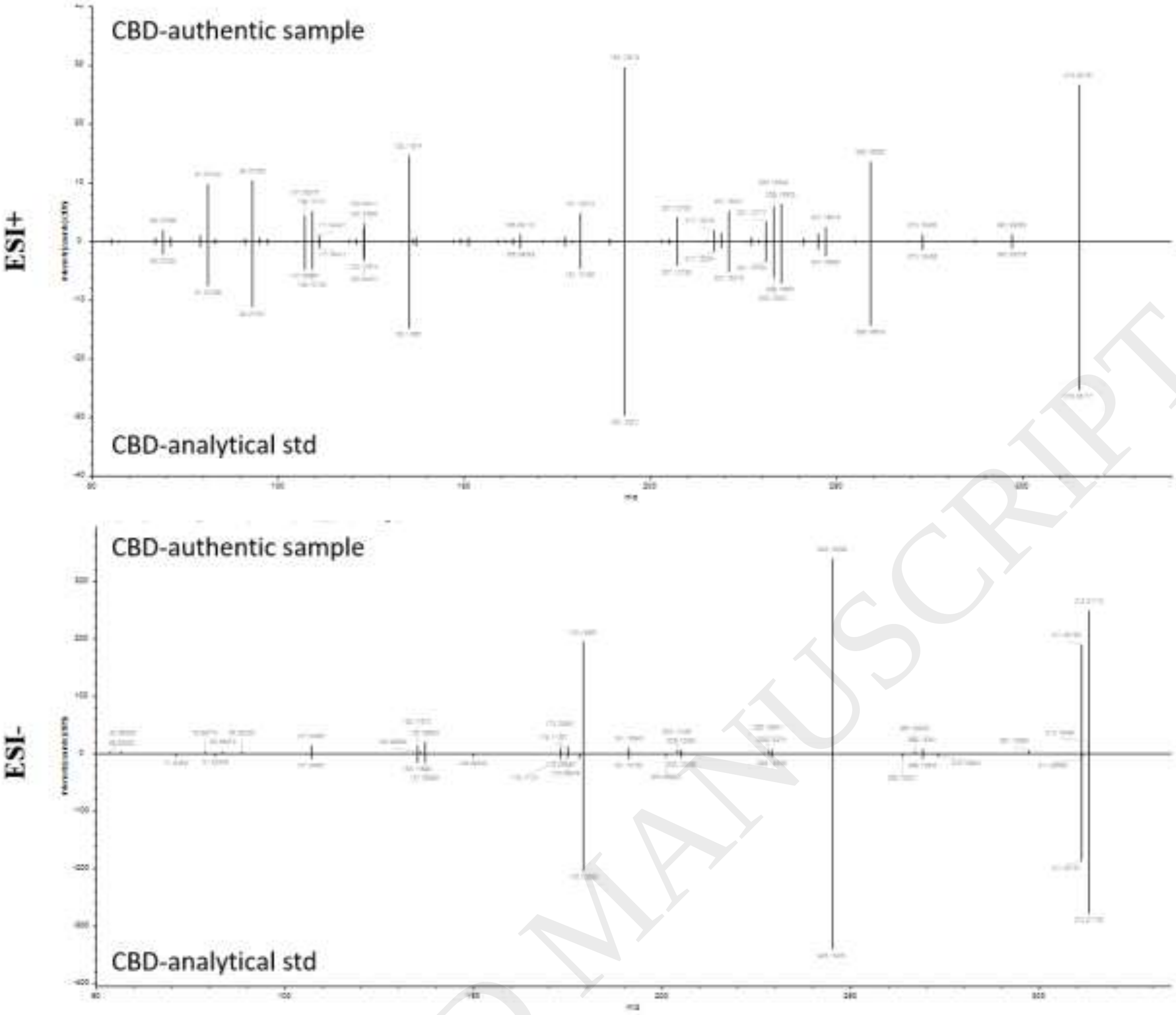


Figure 4. Match of MS/MS spectra of CBD in authentic samples and reference analytical standard in positive (top) and negative (bottom) ionization mode by UHPLC-HESI-Orbitrap.

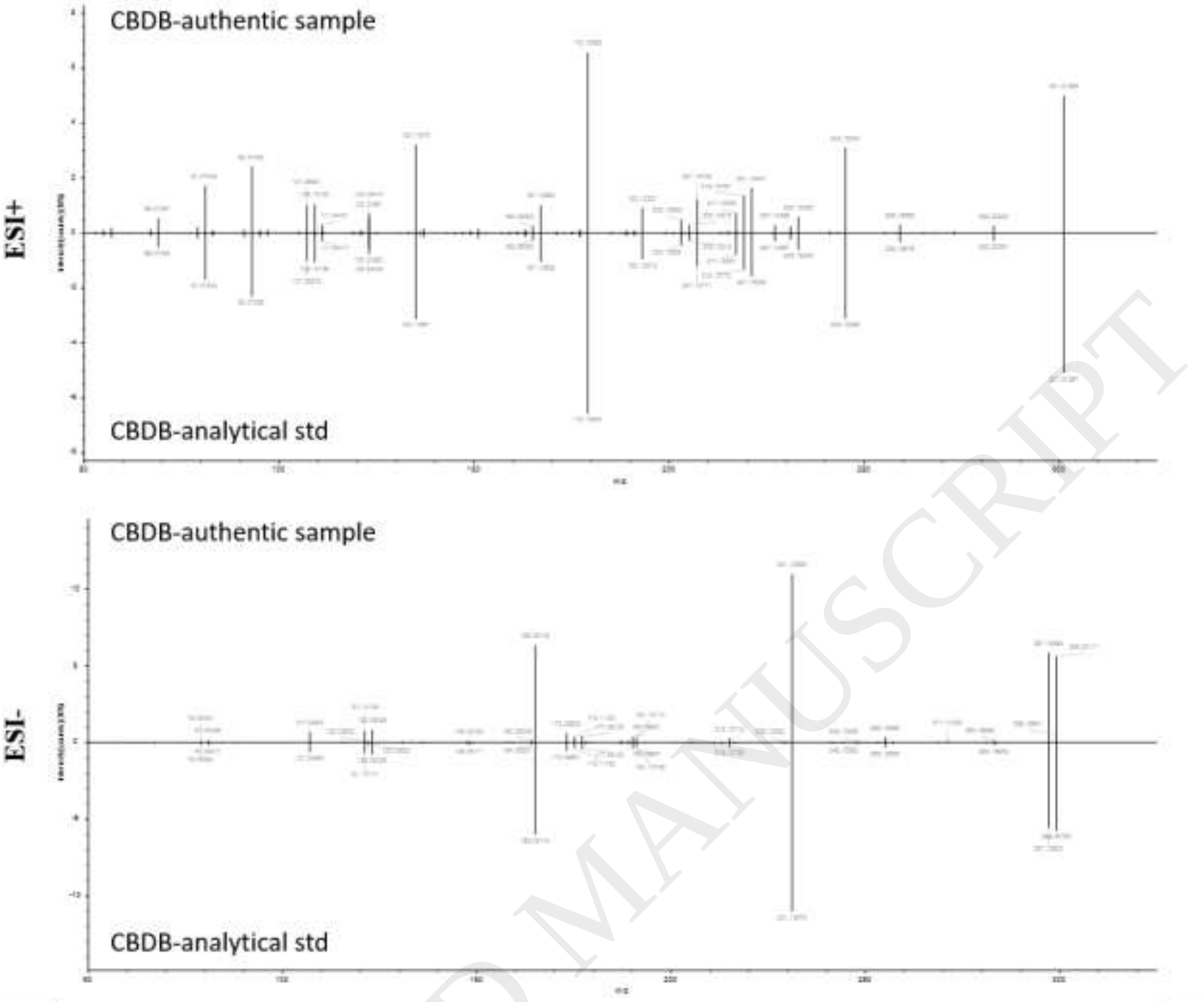


Figure 5. Match of MS/MS spectra of CBDB in authentic samples and reference analytical standard (synthetic CBDB) in positive (top) and negative (bottom) ionization mode by UHPLC-HESI-Orbitrap.

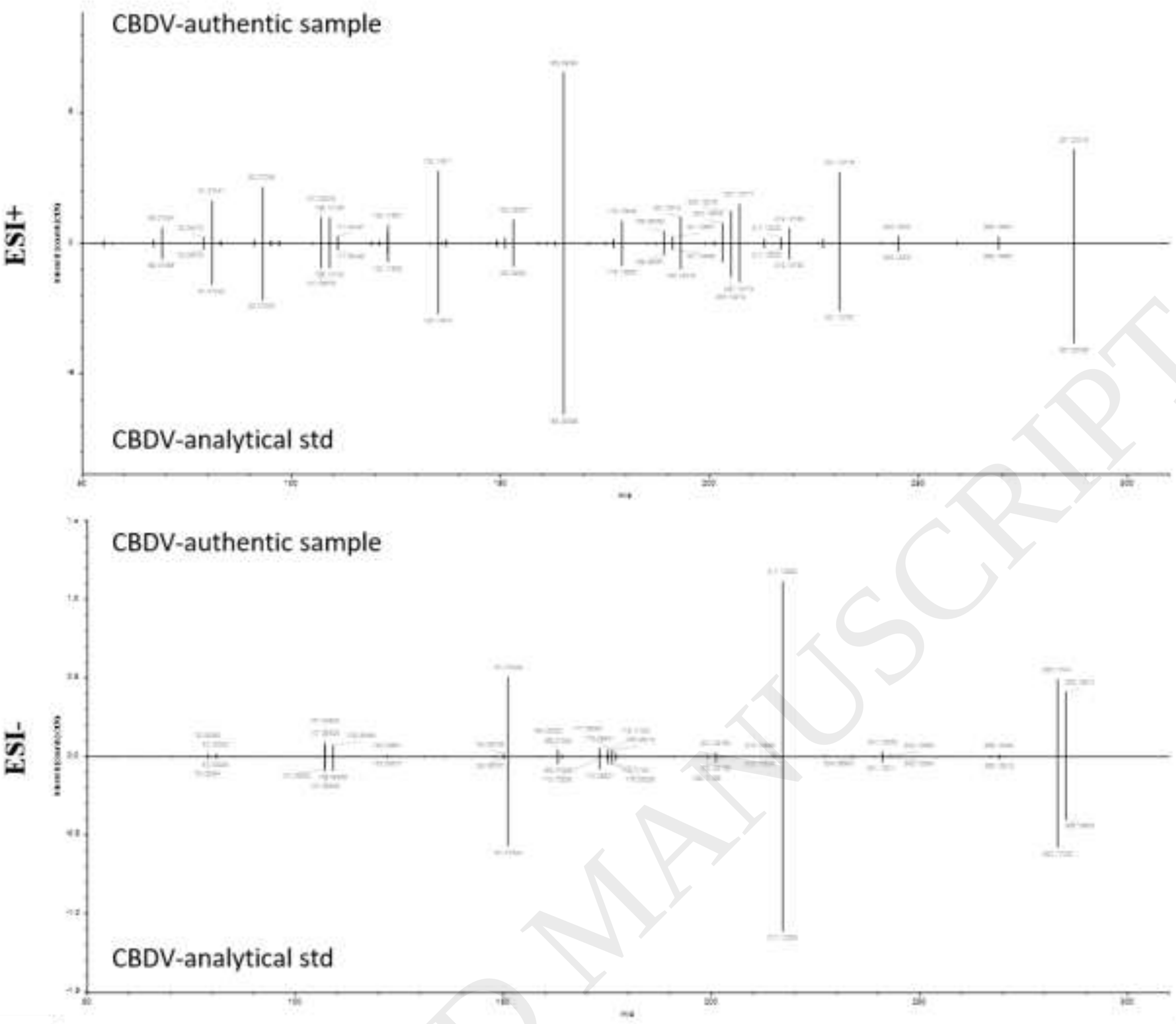


Figure 6. Match of MS/MS spectra of CBDV in authentic samples and reference analytical standard in positive (top) and negative (bottom) ionization mode by UHPLC-HESI-Orbitrap.

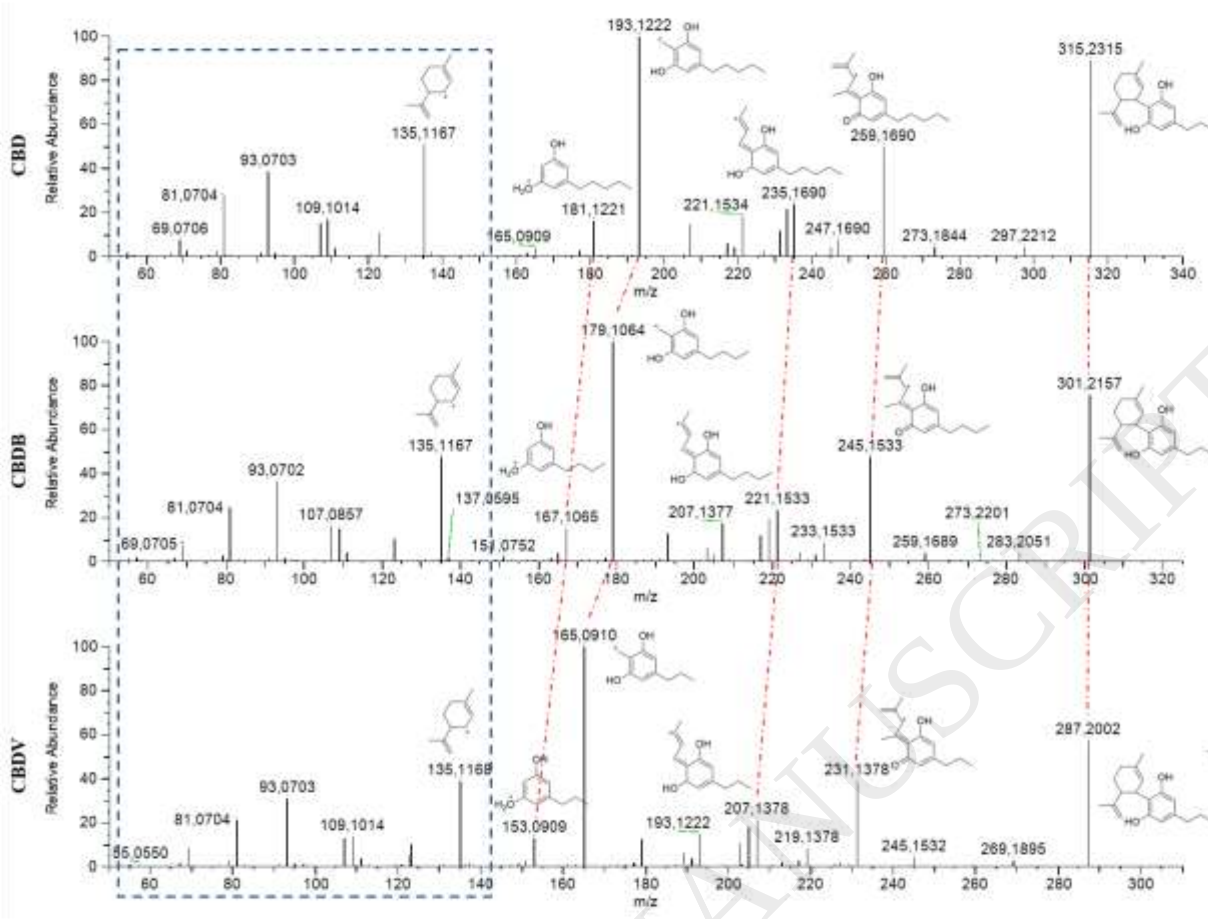


Figure 7. Fragmentation pattern of CBD, CBDB and CBDV in positive ionization mode. The chemical protonated structure is indicated for the main fragments. Red dashed lines indicate the matching fragments between the three cannabinoids. The blue dashed box includes the unchanged fragments belonging to the terpene moiety.

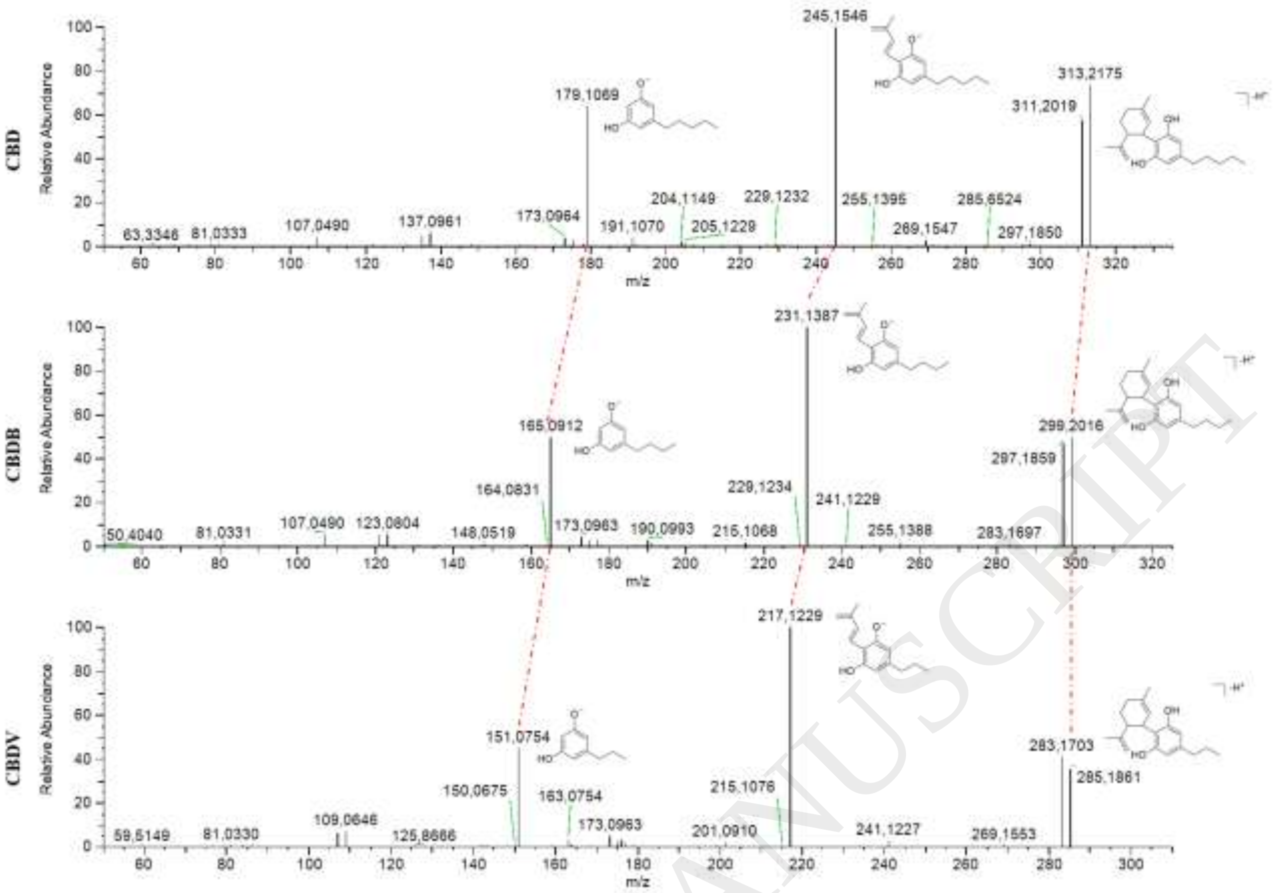


Figure 8. Fragmentation pattern of CBD, CBDB and CBDV in negative ionization mode. The chemical ionized structure is indicated for the main fragments. Red dashed lines indicate the matching fragments between the three cannabinoids.

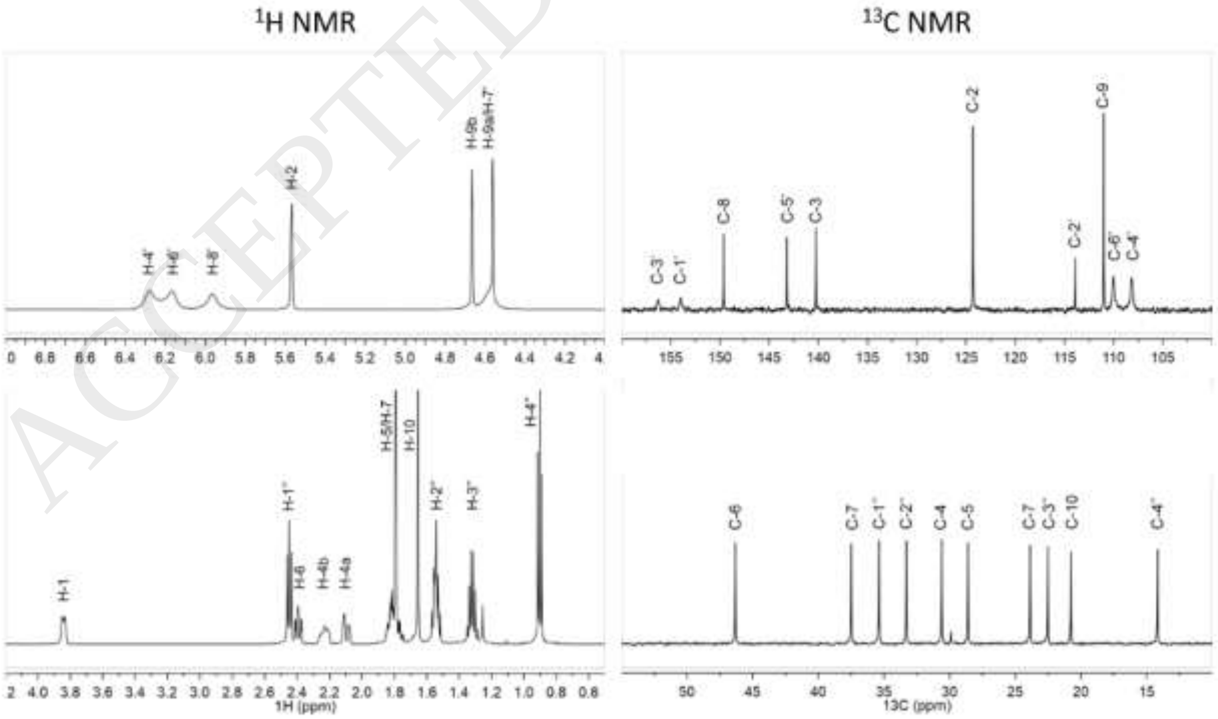


Figure 9. ¹H-NMR (A) and ¹³C-NMR (B) spectra and peaks assignment for CBDB.

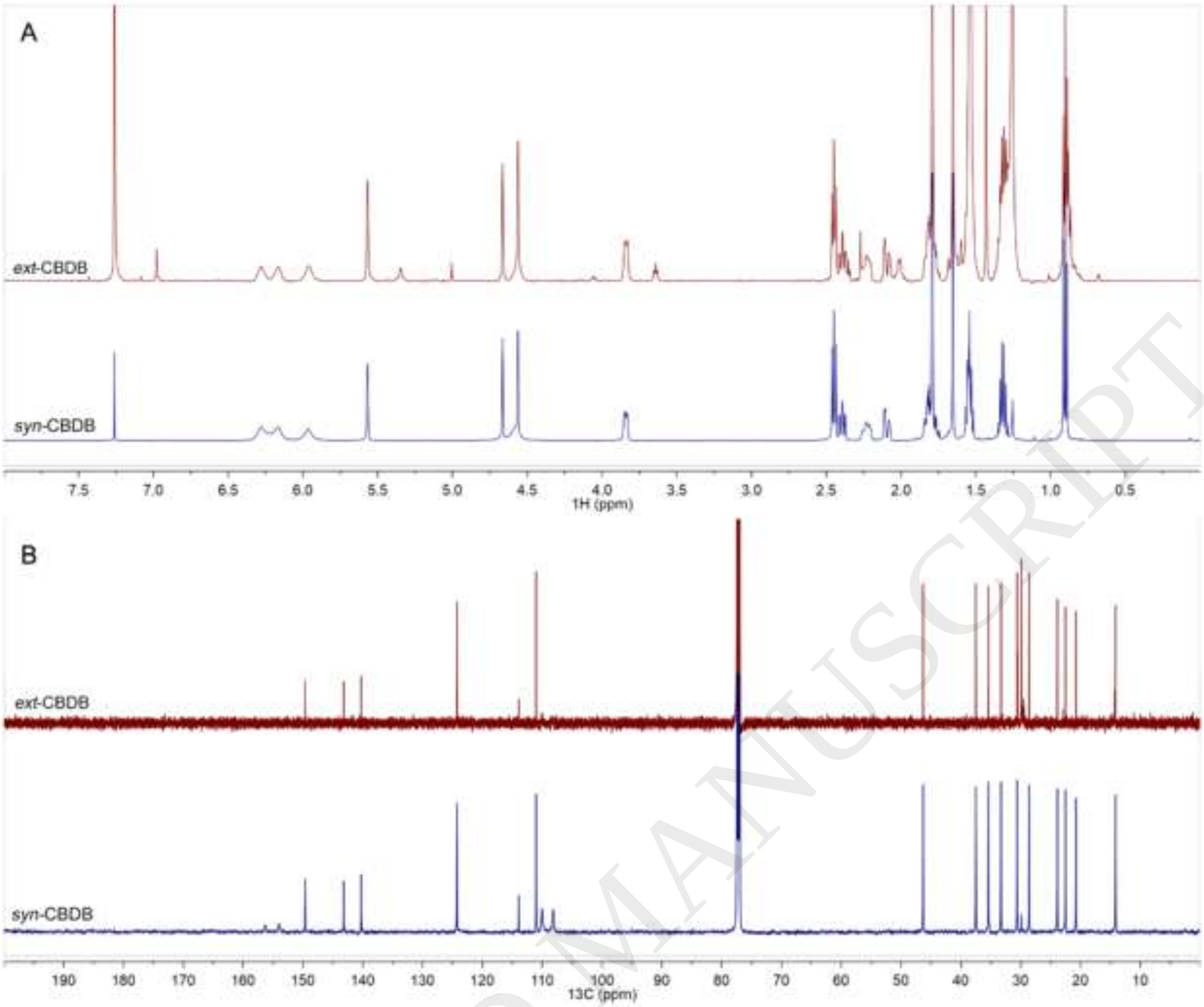
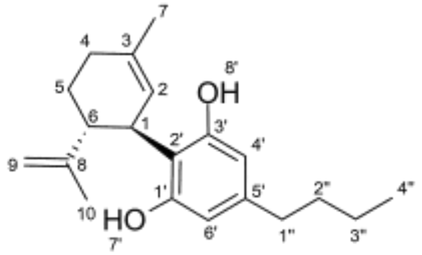


Figure 10. Superimposition of ^{1}H-NMR (A) and ^{13}C-NMR spectra (B) of isolated natural (ext-CBDB, red spectra) and synthesized (syn-CBDB, blue spectra).

Tables

Table 1. *^{1}H and ^{13} NMR assignments (δ) of CBDB[a].*



Position	^{1}H-NMR[b]	^{13}C-NMR
1	3.82-3.86 (m)	37.50
2	5.57 (s)	124.29
3		140.25
4a	2.08-2.12 (m)	30.59
4b	2.22-2.24 (m)	
5	1.76-1.85 (m)	28.60
6	2.40 (dt)	46.32
7	1.79 (s)	23.87
8		149.62
9a	4.56 (s)	111.01
9b	4.67 (s)	
10	1.65 (s)	20.76
1’		154.01
2’		113.92
3’		156.29
4’	6.17 (bs)	108.17
5’		143.19
6’	6.28 (bs)	110.02
7’	4.58 (bs)	
8’	5.97 (s)	
1’’	2.45 (t)	35.37
2’’	1.52-1.57 (m)	33.29
3’’	1.32 (sxt)	22.51
4’’	0.90 (t)	14.15

[a] NMR spectra were recorded in $CDCl_3$ 99.9% of deuteration on a Bruker 600 spectrometer with ^{1}H at 600 MHz and ^{13}C at 151 MHz. Chemical shifts are reported in parts per million (ppm, δ units). Proton chemical shifts were referenced to the solvent residual peak of $CDCl_3$ (7.26 ppm). [b] Splitting patterns are designed as s, singlet; t, triplet; sxt, sextet; dt, double triplet; m, multiplet; b, broad.

Table 2. *Comparison between the chemical shift of the proton signals of the terpene moiety among trans-CBDB, trans-CBD and cis-CBD.*

		^{1}H-NMR (δ)	
Position	***trans*-CBDB**	**(-)-*trans*-CBD**	**(-)-*cis*-CBD**
1	3.82-3.86	3.81-3.84	3.75-3.35
2	5.57	5.54	5.40

6	2.40	2.38	2.90

Table 3. Analysis of authentic commercial CBD samples. The values are expressed as mean percentage (w/w) of three analyses (n=3, standard deviation is not indicated as it was lower than 0.0001 for all samples).

Sample	CBDV (%)	CBDB (%)
CBD-1	0.07	0.08
CBD-2	0.15	0.10
CBD-3	0.34	0.16
CBD-4	0.25	0.13
CBD-5	0.19	0.17
CBD-6	0.17	0.11
CBD-7	0.33	0.16
CBD-8	0.41	0.19
CBD-9	0.33	0.23
CBD-10	0.27	0.22