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The novel heptyl phorolic acid cannabinoids content in different *Cannabis sativa* L. accessions

Pasquale Linciano [a,b], Fabiana Russo [a], Cinzia Citti [a,c,*], Francesco Tolomeo [c], Roberta Paris [d], Flavia Fulvio [d], Nicola Pecchioni [e], Maria Angela Vandelli [a], Aldo Laganà [c,f], Anna Laura Capriotti [f], Giuseppe Biagini [g], Luigi Carbone [c], Giuseppe Gigli [c], Giuseppe Cannazza [a,c]

[a] Department of Life Sciences, University of Modena and Reggio Emilia, Via G. Campi 103, 41125, Modena, Italy
[b] School of Pharmacy, University of Pavia, Viale Taramelli 12, 27100, Pavia, Italy
[c] Institute of Nanotechnology, CNR NANOTEC, Via Monteroni, 73100, Lecce, Italy
[d] CREA-Research Center for Cereal and Industrial Crops, Via di Corticella 133, 40128, Bologna, Italy
[e] CREA-Research Center for Cereal and Industrial Crops, S.S. 673 Km 25,200, 71122, Foggia, Italy
[f] Department of Chemistry, Sapienza University of Rome, Piazzale Aldo Moro 5, 00185, Rome, Italy
[g] Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Via G. Campi 287, 41125, Modena, Italy

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ABSTRACT

The recent discovery of the novel heptyl phytocannabinoids cannabidiphorol (CBDP) and Δ^9-tetrahydrocannabiphorol (Δ^9-THCP) raised a series of questions relating to the presence and abundance of these new unorthodox compounds in cannabis inflorescence or derived products. As fresh inflorescence contains mainly their acid precursors, which are not commercially available, an *ad hoc* stereoselective synthesis was performed in order to obtain cannabidiphorolic acid (CBDPA) and Δ^9-tetrahydrocannabiphorolic acid (THCPA) to be used as analytical standards for quantitative purposes. The present work reports an unprecedented targeted analysis of both pentyl (C5) and heptyl (C7) CBD- and THC-type compounds in forty-nine cannabis samples representing four different chemotypes. Moreover, the ultrahigh performance liquid chromatography coupled to high-resolution mass spectrometry-based method was applied for the putative identification of other heptyl homologs of the most common phytocannabinoid acids, including cannabigerophorolic acid (CBGPA), cannabichromephorolic acid (CBCPA), cannabinophorolic acid (CBNPA), cannabielsophorolic acid (CBEPA), cannabicyclophorolic acid (CBLPA), cannabitriophorolic acid (CBTPA), and cannabiripsophorolic acid (CBRPA).

1. Introduction

Cannabis research has made great progresses in the last few years thanks to a renewed interest in this plant from both public institutions and private companies. The attention towards cannabis is particularly due to the well-known class of phytocannabinoids, which includes the non-psychotropic cannabidiol (CBD) and the psychotropic Δ^9-tetrahydrocannabinol (Δ^9-THC) (Fig. 1). Since their discovery in the early 60s by Mechoulam and co-workers [1,2], the inventory of phytocannabinoids has grown to a very large extent reaching a number slightly below 150 [3]. In this regard, in the last two years six new phytocannabinoids, homologs of CBD and Δ^9-THC, have been discovered, tearing down previous beliefs on the chemistry of cannabis [4–8]. The new homologs differ from canonical compounds in the length of the alkyl side chain on the resorcinyl group. In particular, cannabidibutol (CBDB) [4–6] and Δ^9-tetrahydrocannabutol (Δ^9-THCB) [6] present a four-term linear (-CH_2 $(CH_2)_2CH_3$) side chain, while cannabidihexol (CBDH) [8] and Δ^9-tetrahydrocannabihexol (Δ^9-THCH) [8] share a *n*-hexyl (-CH_2 $(CH_2)_4CH_3$) side chain, and cannabidiphorol (CBDP) [7] and Δ^9-tetrahydrocannabiphorol (Δ^9-THCP) [7] are characterized by a seven-term linear (-$CH_2(CH_2)_5CH_3$) side chain (Fig. 1). All these new phytocannabinoids derive from their acid precursors cannabidibutolic acid (CBDBA), tetrahydrocannabutolic acid (THCBA), cannabidihexolic acid (CBDHA), tetrahydrocannabihexolic acid (THCHA), cannabidiphorolic acid (CBDPA), and tetrahydrocannabiphorolic acid (THCPA), as they were putatively identified in samples of cannabis inflorescence [6–8].

* Corresponding author. Institute of Nanotechnology, CNR NANOTEC, Via Monteroni, 73100, Lecce, Italy.
E-mail addresses: cinzia.citti@unimore.it, cinzia.citti@nanotec.cnr.it (C. Citti).

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Whilst the biological activity of CBD homologs has still to be evaluated, with the exception of the analgesic activity of CBDH [8], Δ^9-THCB [6] and Δ^9-THCP [7] were tested in both *in vitro* and *in vivo* assays. In details, preliminary investigations indicated that Δ^9-THCB has an affinity for CB_1 receptors similar to that of Δ^9-THC and a partial agonistic activity in behavioural tests [6]. On the other hand, Δ^9-THCP showed a surprisingly 30-fold affinity for CB_1 receptors compared to Δ^9-THC and an *in vivo* cannabimimetic activity similar to Δ^9-THC but at half the dose [7]. Besides the bewildering biological activity of Δ^9-THCP, a natural cannabinoid with a side chain longer than five carbon atoms has never been reported nor even hypothesized until now, and these findings have baffled the scientific community on this topic [7]. As a result, these studies have paved the way to the identification of a new series of phytocannabinoids, the butyl [6], hexyl [8] and heptyl homologs [7] of CBD and THC, but have also disclosed the existence of a phytocannabinoid in a cannabis variety with a psychotropic activity potentially higher than THC, hitherto considered the main psychotropic constituent of cannabis.

In our recent work, the identification of both CBDP and Δ^9-THCP was accomplished after thermal decarboxylation of their putative acidic precursors, namely cannabidiphorolic acid (CBDPA) and Δ^9-tetrahydrocannabiphorolic acid (Δ^9-THCPA), which bear a carboxylic group on the resorcinyl moiety similarly to all phytocannabinoid precursors [7]. The experiments were performed on the medicinal cannabis variety FM2 bred as CINRO by CREA-CI (Research Centre for Cereal and Industrial Crops) site in Rovigo (Italy) and supplied by the Military Chemical Pharmaceutical Institute (Florence, Italy) [7]. It is known that the decarboxylation process can lead to a degradation of the phytocannabinoid molecules, thus not reflecting the actual concentrations of the original compounds in the plant [9–11].

The unambiguous identification of a new phytocannabinoid requires the confirmation of the retention time, mass to charge ratio (*m/z*), and MS/MS spectrum match with a pure analytical standard. As these novel phytocannabinoids are not commercially available, an *in house* stereoselective synthesis reported in the present work allowed to confirm our putative identification of CBDPA and Δ^9-THCPA in the FM2 cannabis variety. On the other hand, the finding of new phytocannabinoids opened important questions: are they present in other cannabis accessions? If so, what are their actual concentrations? The present work aims to answer these open questions through the analysis of samples collected from forty-nine cannabis accessions provided by CREA-CI (Rovigo, Italy) and belonging to I-IV chemotypes [12] by an ultrahigh performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS) method. Our research was not only limited to the determination of CBDPA and THCPA, but also to the putative identification of other species of this series of phytocannabinoids, such as cannabigerophorolic acid (CBGPA), cannabichromephorolic acid (CBCPA), and cannabielsophorolic acid (CBEPA), cannabiripsophorolic acid (CBRPA), cannabitriophorolic acid (CBTA), cannabicyclophorolic acid (CBLPA), and cannabinophorolic acid (CBNPA) (Fig. 1).

2. Materials and methods

2.1. Materials

Analytical grade ethanol 96% (Carlo Erba) was used for the extraction of the various cannabis inflorescence samples. LC-MS grade acetonitrile, water and formic acid were purchased from Carlo Erba (Milan, Italy) and employed in the UHPLC-HRMS analyses. Δ^9-Tetrahydrocannabinol (Δ^9-THC), Δ^9-tetrahydrocannabinolic acid (THCA), cannabidiol (CBD), cannabidiolic acid (CBDA), cannabichromenic acid (CBCA), and cannabigerolic acid (CBGA) were purchased as Cerilliant certified analytical standards (Sigma-Aldrich, Milan, Italy). Cannabidiphorol (CBDP) and Δ^9-tetrahydrocannabiphorol (Δ^9-THCP) were prepared following our *in house* synthesis, as previously reported [7]. Reagents and solvents for synthesis were purchased from Sigma-Aldrich and used as arrived, unless otherwise specified. Organic solvents were abbreviated as follows: chloroform ($CHCl_3$); deuterium-chloroform ($CDCl_3$); cyclohexane (CE); dichloromethane (DCM); diethyl ether (Et_2O); dimethyl formamide (DMF).

2.2. Synthesis and characterization of CBDPA and THCPA

Reaction monitoring was performed by thin-layer chromatography on silica gel (60F-254, E. Merck) and checked by UV light or alkaline $KMnO_4$ aqueous solution. Reaction products were purified by flash chromatography on silica gel (40–63 μm) with the solvent system indicated. NMR spectra were recorded on a Bruker 400 spectrometer working at 400.134 MHz for ^1H and at 100.62 MHz for ^{13}C. Chemical shifts (δ) are reported in parts per million (ppm) and they were referenced to the solvent residual peaks ($CDCl_3$ $\delta = 7.26$ ppm for proton and $\delta = 77.20$ ppm for carbon); coupling constants are reported in hertz (Hz); splitting patterns are expressed with the following abbreviations: singlet (s), doublet (d), triplet (t), quartet (q), double doublet (dd), quintet (qnt), multiplet (m), broad signal (b). COSY spectra were recorded as a 2048 × 256 matrix with 2 transients per t1 increment and processed as a 2048 × 1024 matrix; the HSQC spectra were collected as a 2048 × 256 matrix with 4 transients per t1 increment and processed as a 2048 × 1024 matrix, and the one-bond heteronuclear coupling value was set to 145 Hz; the HMBC spectra were collected as a 4096 × 256 matrix with 16 transients per t1 increment and processed as a 4096 × 1024 matrix, and the long-range coupling value was set to 8 Hz.

Optical rotation (α) was measured with a Polarimeter 240C (cell-length 100 mm, volume 1 mL) from PerkinElmer (Milan, Italy).

2.3. Plant material and sample preparation

The female or monoecious inflorescences collected at maturity from

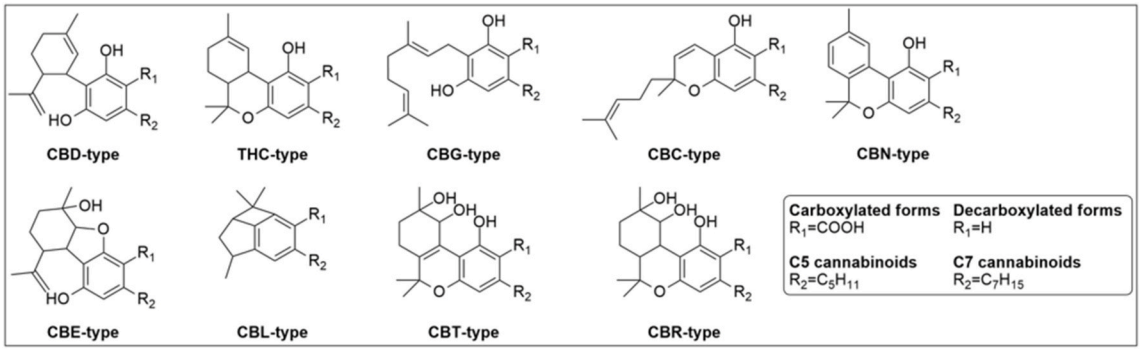


Fig. 1. General chemical structure of C5 and C7 phytocannabinoids. General chemical structure of C5 and C7 acid (carboxylated) and neutral (decarboxylated) phytocannabinoids, including CBD-, THC-, CBG-, CBC-, CBN-, CBE-, CBL-, CBT-, and CBR-type compounds.

forty-nine cannabis varieties/accessions were obtained from *Cannabis* germplasm collection available at the Research Centre for Cereal and Industrial Crops (CREA-CI) in Rovigo (Italy). In particular, seventeen samples were female inflorescences collected from chemotype I plants, with high THC levels (and poor in other cannabinoids); six samples were from chemotype II genotypes, with a balanced level of THC and CBD; twenty samples were from chemotype III varieties/accessions with a high CBD content (and low content of other cannabinoids); and six samples taken from chemotype IV genotypes, characterized by a prevalence of CBG. Chemotype V varieties were not taken into consideration as they have amounts of cannabinoids close to zero. More details on the genotypes (geographical origin, designation and use, harvest date, plant material, sex, drying process, cultivation) are given in Table S1 (Appendix A, Supplementary Material). Varieties are indicated with their names while other accessions are indicated with a code starting with S or a V depending whether they are propagated by seed or vegetatively. Cultivation of *Cannabis sativa* L. plants for scientific purposes on field areas and greenhouses with THC limit 5% and 10% respectively was authorized according to art. 26 of the D.P.R. 309/90 (authorization n. SP/052, March 31, 2017); indoor cultivations of cannabis plants with THC limit of 25% were granted with authorization n. SP/041 on March 13, 2017 according to art. 26 of the D.P.R. 309/90. Cultivation started in March 2018 and the harvesting was concluded in November 2018.

Samples (2 g each) were finely ground and extracted in ethanol 96% according to the monograph of *Cannabis flos* of the German Pharmacopoeia and as previously reported in other works (500 mg in 50 mL of solvent) [7,13–15]. For the determination of the pentyl phytocannabinoids the samples were 1000-fold diluted with mobile phase, while the heptyl homologs were determined by diluting the sample 10-fold.

2.4. UHPLC-HRMS analysis

The analysis of the samples was carried out on a Thermo Fisher Scientific Ultimate 3000 UHPLC system provided with a vacuum degasser, a binary pump, a thermostated autosampler set at 4 °C, and a thermostated column compartment set at 25 °C. The chromatographic apparatus was interfaced to a heated electrospray ionization source and a Q-Exactive Orbitrap mass spectrometer (UHPLC-HESI-Orbitrap). For an optimal detection of the species under investigation, the parameters of the HESI source employed in our previous work were applied: 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 [4]. The analyses were acquired with Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) in full scan data-dependent acquisition (FS-dd-MS^2) in positive (HESI+) and negative (HESI-) mode at a resolving power of 70,000 FWHM at *m/z* 200; the scan range was set in the window of *m/z* 150–750 to achieve a higher sensitivity for the molecular weight of cannabinoids, the AGC target was set at 3e6, the injection time at 100 ms and the isolation window for the filtration of the precursor ions at *m/z* 0.7 to improve selectivity [4]. A collision energy of 20 eV was used to fragment the precursor ions. $[M+H]^+$ and $[M-H]^-$ molecular ions were extracted from the total ion chromatogram (TIC) of each extracts and matched with pure analytical standards for accuracy of the exact mass ($\Delta = 5$ ppm), retention time ($\Delta = 0.1$ min) and MS/MS spectrum.

The chromatographic separation was carried out on a column with a core-shell based stationary phase (Poroshell 120 SB-C18, 3 × 100 mm, 2.7 μm, Agilent, Milan, Italy) and a mobile phase composed of 0.1% (v/v) aqueous formic acid (A) and acetonitrile (B) following the conditions employed in our previous work with a linear gradient from 70 to 98% B (0–25 min), an isocratic elution with 98% B (25.1–28.0) min, and a final re-equilibration with 70% B (28.1–30.0 min) [4,16].

A semi-quantitative analysis of CBD, Δ^9-THC, CBDP, Δ^9-THCP, CBDA, THCA, CBDPA, and THCPA was achieved by building the corresponding calibration curves using external standards. A stock solution of all analytes (1 mg/mL) was properly diluted to obtain five non-zero calibration points at the final concentrations of 10, 50, 100, 500 and 1000 ng/mL. The linearity was assessed by the coefficient of determination (R^2), which was greater than 0.997 for each analyte.

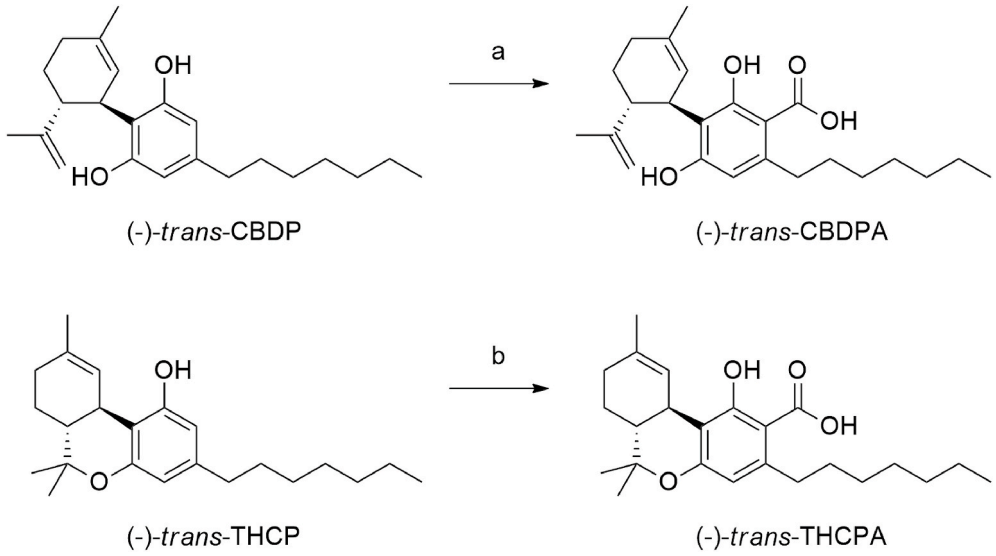
2.5. Statistical analysis

Data have been compared by one-way analysis of variance (ANOVA) followed by Bonferroni's correction for multiple comparisons. All the analyses were performed with GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). Results are illustrated by mean values and standard errors of the mean and considered significant at $p < 0.05$.

3. Results and discussion

3.1. Synthesis and characterization of CBDPA and THCPA

Mechoulam already reported the chemical conversion of (−)-*trans*-CBD and (−)-*trans*-Δ^9-THC into their corresponding acid (carboxylated) forms [17]. Accordingly, in the present study, the synthesis of the analytical standards of (−)-*trans*-CBDPA and (−)-*trans*-Δ^9-THCPA was performed starting from the corresponding neutral (decarboxylated) cannabinoids (−)-*trans*-CBDP and (−)-*trans*-Δ^9-THCP, which were prepared as previously reported by our research group [7]. As reported in Scheme 1, (−)-*trans*-CBDP and (−)-*trans*-Δ^9-THCP were reacted in a Kolbe-like reaction, using 2 M methyl magnesium carbonate (MMC) in DMF as carboxylating agent, at 120–130 °C for 3 h (for (−)-*trans*-CBDPA) or overnight (for (−)-*trans*-Δ^9-THCPA). Of note, the reaction was performed in close vessels in order to avoid the loss of carbon dioxide thus promoting the shift of the reaction equilibrium towards the product. The two standards were obtained in 39% and 7% yield for (−)-*trans*-CBDPA and (−)-*trans*-Δ^9-THCPA, respectively, and the yields are in line with those reported by Mechoulam [17]. One of the main issues related to the acid forms of CBDs and THCs is their poor chemical stability, since they easily and spontaneously undergo decarboxylation, especially by heating. It was therefore necessary to set up a protocol to purify the CBDPA and THCPA from the unreacted starting materials and by-products, in particular using low-boiling solvents that could be evaporated at low temperature and under modest reduced pressure. CBDPA and THCPA were purified by solid phase extraction on silica gel. The reaction crude was eluted first using a mixture of CE/DCM 1:1 to remove the unreacted starting materials and by-products. In these conditions, CBDPA and THCPA were completely retained by silica gel. Thereafter, by elution with 100% Et_2O it was possible to recover the sole desired product. Moreover, since Et_2O is a very low-boiling solvent (boiling temperature 34.6 °C) it was removed at room temperature without affecting the stability of the final compounds that were thus achieved in high purity (>98%) as stated by UHPLC-UV/MS analysis. The chemical identification of synthetic (−)-*trans*-CBDPA and (−)-*trans*-Δ^9-THCPA was confirmed by NMR spectroscopy and HRMS. The monodimensional (^{1}H and ^{13}C) and bidimensional (COSY, HSQC and HMBC) NMR spectra are reported in the Material and Methods section and in Appendix B (Supplementary Material). Since (−)-*trans*-CBDPA and (−)-*trans*-Δ^9-THCPA differ from the respective homologs (−)-*trans*-CBDA and (−)-*trans*-Δ^9-THCA solely for the length of the alkyl chain on the resorcinyl moiety, no significant differences in the proton and carbon chemical shifts of the terpene and aromatic moieties were expected. The perfect match in the chemical shift of the terpene and aromatic moieties between the synthesized (−)-*trans*-CBDPA and (−)-*trans*-Δ^9-THCPA and the respective homologs (−)-*trans*-CBDA and (−)-*trans*-Δ^9-THCA [18], combined with the HRMS spectra and fragmentation pattern, allowed us to unambiguously confirm the chemical structures of the two new synthetic cannabinoids. Lastly, the stereochemistry of the starting materials (−)-*trans*-CBDP and (−)-*trans*-Δ^9-THCP was fully investigated and confirmed in our previous study [8]. We were confident that the synthetic conditions adopted for



Scheme 1. Reagents and conditions: a) 2 M MMC in DCM, 120 °C, 3 h. b) 2 M MMC in DCM, 130 °C, 18 h.

the synthesis of the corresponding acids did not affect the absolute configuration of the two stereocenters, and the position of the double bond. For these reasons, we could confirm the Δ^9 position of the double bond and the *1′R,2′R* and *6aR, 10aR* absolute configuration for (−)-*trans*-CBDPA and (−)-*trans*-Δ^9-THCPA, respectively. Description of the synthetic procedure and characterization of the two new compounds are reported in Appendix B (Supplementary Material).

3.2. Identification of CBDPA and THCPA

The acidic precursors of CBDP and Δ^9-THCP, namely cannabidiphorolic acid (CBDPA) and tetrahydrocannabiphorolic acid (THCPA), were putatively identified and reported in a previous paper [7]. In the present work, these phytocannabinoid acids were identified in the forty-nine varieties by means of high-resolution Orbitrap mass spectrometry and confirmed by match with pure synthesized standards. In details, CBDPA and THCPA presented the same molecular ions in both positive and negative ionization mode with $[M+H]^+$ at *m/z* 387.2530 and $[M - H]^-$ at *m/z* 385.2384, respectively, and molecular formula $C_{24}H_{34}O_4$. CBDPA and THCPA eluted at 18.78 min and 22.18 min respectively (Fig. 2). As expected, the fragmentation spectra of the two compounds were identical in positive ionization mode (HESI+), while they could be distinguished in negative ionization mode (HESI-). In HESI + mode, the molecular ion was barely visible, as well as the fragment corresponding to the loss of the carboxylic moiety at *m/z* 341; the loss of water, instead, generated the base peak at *m/z* 369. The fragment at *m/z* 289 was produced by the loss of water and part of the terpene moiety, followed by the complete loss of the latter to generate the fragment at *m/z* 247. On the other hand, the relative abundance of the fragments in HESI- mode was diagnostic of the cannabinoid type since CBD-type cannabinoids generally present a fragment rich spectrum, whereas THC-type spectra are usually characterized by a reduced number of fragment peaks. The only common features were the base peak, which corresponded to the precursor ion, and the fragment generated by the loss of CO_2 at *m/z* 341. The loss of water generated the fragment at *m/z* 367, which was visible only in the spectrum of CBDPA. The fragment at *m/z* 273, higher in the spectrum of CBDPA, was produced by the loss of water and part of the terpene moiety, while the fragment at *m/z* 207, lacking the whole terpene moiety, was observed only in the CBDPA spectrum. Fig. 2 shows the match of retention times in both HESI+ and HESI- mode of CBDPA and THCPA in a synthetic standard mixture (100 ng/mL) and in a real sample (from the medical variety CINRO cultivated indoor); comparison of the high-resolution mass fragmentation spectra of the two compounds in both ionization modes are also reported. Fragmentation patterns of both C7 phytocannabinoid acids confirmed the data previously reported for putatively identified CBDPA and THCPA [16].

3.3. Putative identification of heptyl phytocannabinoid acids

The discovery of CBDPA and THCPA in the pharmaceutical cultivar CINRO [7] prompted the research of other phytocannabinoids of the heptyl series. Therefore, the possible existence of cannabigerophorolic acid (CBGPA), cannabichromephorolic acid (CBCPA), cannabielsophorolic acid (CBEPA), cannabicyclophorolic acid (CBLPA), cannabitriophorolic acid (CBTPA), and cannabiripsophorolic acid (CBRPA), heptyl homologs of the most common phytocannabinoid acids, was further investigated (Fig. 1).

The corresponding precursor ions were searched in both HESI+ and HESI- mode, but only two of the aforementioned compounds were detected and only in HESI- mode, which is generally more sensitive for carboxylated cannabinoids. Due to the likely scarce abundance of such homologs, it was not possible to detect the HESI + peak and HRMS spectra. The $[M - H]^-$ ion at *m/z* 387.2546 corresponding to the chemical formula $C_{24}H_{36}O_4$ was putatively identified as CBGPA given the perfect correspondence of the fragments and relative abundance in the HRMS spectrum with its pentyl homolog cannabigerolic acid (CBGA) with the addition of two methylene units (28.0313 amu), as shown in Figure S1 (Appendix B, Supplementary Material). CBGPA precursor ion $[M - H]^-$ at *m/z* 387 is considerably higher than that of CBGA at *m/z* 359. Such phenomenon occurs also for the other C7 species like THCPA and CBDPA, for which the precursor ion exceeds by approximately twice that of their C5 homologs THCA and CBDA, most likely because C7 precursor ion needs higher collision energies for a stronger fragmentation. The loss of water produces the base peak for both CBGA and CBGPA at *m/z* 341 and 369 respectively. Besides the fragment generated by the loss of CO_2 at *m/z* 315 and 343 for CBGA and CBGPA respectively, the other fragments showed very low abundance.

In the same way, CBCPA was putatively identified from its $[M - H]^-$ molecular ion and chemical formula, which are identical to those of CBDPA and THCPA. Moreover, the HRMS spectrum of the putative CBCPA was comparable to that of its pentyl homolog cannabichromenic acid (CBCA) with two additional methylene units (Figure S2, Appendix B, Supplementary Material).

The highest signal for the peak area of CBGPA and CBCPA was registered in the sample from the female inflorescence of the

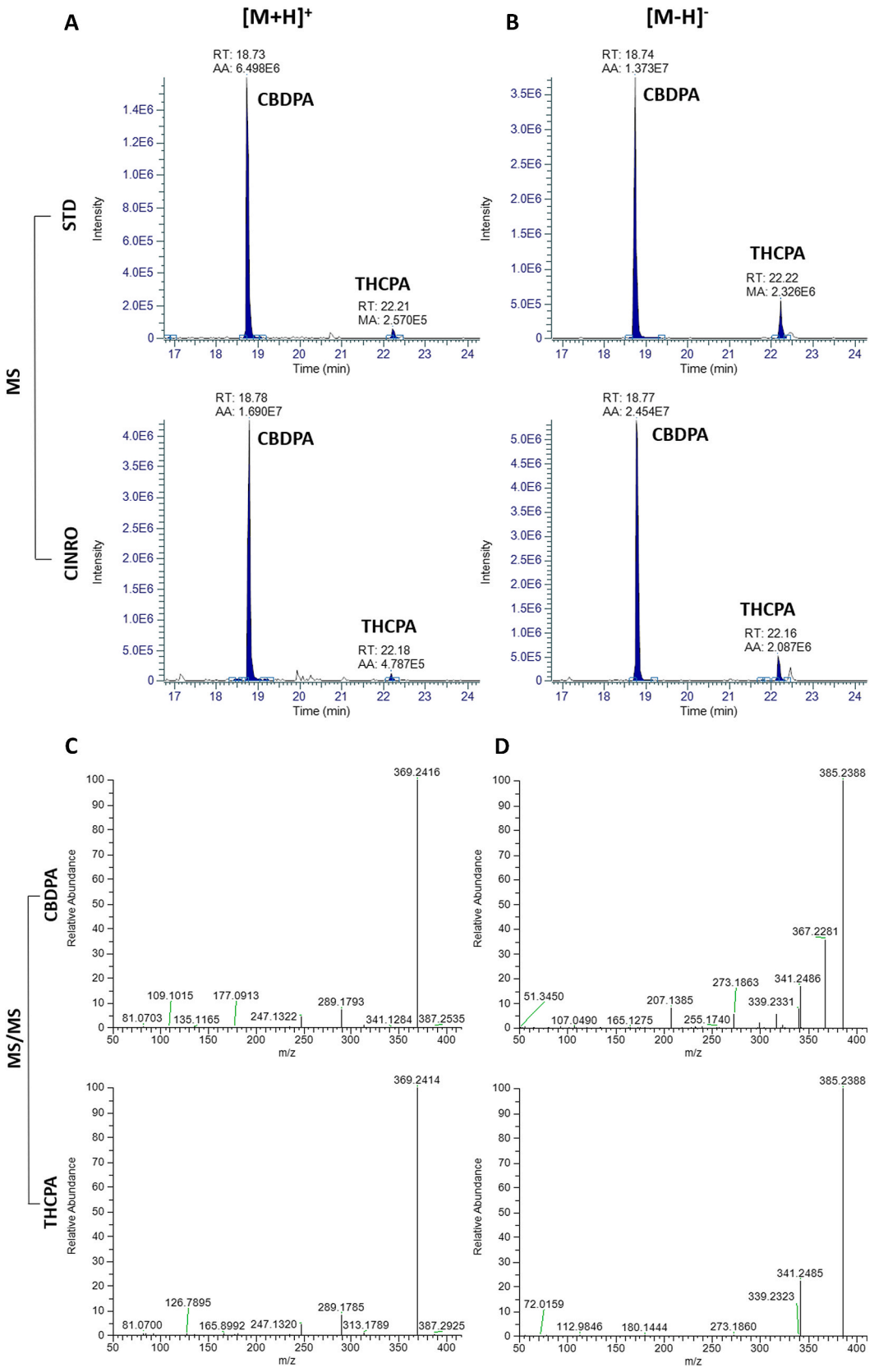


Fig. 2. Match of standard and natural CBDPA and THCPA. HPLC-HRMS chromatograms of standard CBDPA and THCPA obtained by stereoselective synthesis and the same compounds found in a cannabis variety (CINRO) in both positive (A) and negative (B) ionization mode. Tandem HRMS fragmentation spectra of CBDPA and THCPA in both positive (C) and negative (D) ionization mode.

experimental accession "V_02". The other heptyl phytocannabinoid acids were not found, although all the pentyl homologs, cannabiripsolic acid (CBRA), cannabitriolic acid (CBTA) and cannabielsoic acid (CBEA), with the exception of cannabicyclolic acid (CBLA), were detected based on the putative identification reported in the literature [14,19].

3.4. Semi-quantification of pentyl and heptyl phytocannabinoids

The synthesized pure standards, obtained with a purity greater than 98%, were used to build the calibration curves for the semi-quantification of CBDPA and THCPA. Moreover, the authentic standards of CBDP and Δ^9-THCP were available from previous *in house* synthesis [7]. These calibrations allowed to provide a reasonably accurate measure of the concentration of the new heptyl phytocannabinoids, along with their pentyl counterparts CBDA, THCA, CBD, and Δ^9-THC for which the pure analytical standards are commercially available. Δ^8-THC was not detected. According to their optimal ionization parameters, the phytocannabinoid acids were determined in the cannabis samples in HESI- mode, whereas the decarboxylated phytocannabinoids were quantified in HESI + mode. Good coefficients of linear correlation were obtained in the range 10–1000 ng/mL for all analytes. The results were obtained from the analysis of three replicates for each sample and summarized per chemotype in Fig. 3. Data are graphed as micrograms of phytocannabinoid per gram of plant material. Detailed data of phytocannabinoids concentrations in each cannabis accession is given in Appendix B (Supplementary Material, Figures S3 and S4).

THCPA was present in half of the samples in concentrations above 100 μg/g reaching the highest amount of 446 μg/g in the CREA experimental accession "V_08–2018" (chemotype I) and 433 μg/g in the variety "CINRO" (chemotype II) ($p < 0.001$, Figure S4). CBDPA reached the highest concentration value of about 1030 μg/g in the floral sample taken from accession "V_02" ($p < 0.001$, Figure S3), which has a chemotype III. Both accessions were selected at CREA-CI for pharmaceutical purposes, with V_08 bearing a prevalence of THCA and V_02 a prevalence of CBDA. These samples showed a proportion of heptyl cannabinoids in the total of main C5 and C7 cannabinoid fraction equal to 0.95% and 0.89% respectively (calculated as C7/(C5+C7)). The highest amount of C7 THC and CBD homologs was found in the female inflorescence of CINRO medical variety (cultivated indoor) with about 1221 μg/g, equal to 1.20% out of the total amount of C5 and C7 cannabinoids and with 67.8% of purity in total CBDP (CBDP/C7) ($p < 0.001$). This variety is a chemotype II with THCA + CBDA = 15% in a 2:3 ratio. Instead, the highest proportion of C7 homologs was found in samples from chemotype I Chinese landraces (S1770, S1605, S1639, and V_11), with a mean value of 1.18% of C7/(C5+C7) cannabinoids ($p < 0.001$). Among the European hemp varieties with chemotype III, the proportion of heptyl homologs ranged from 0.23% to 0.55%. In the same group, the average of total CBD and total THC concentrations showed a similar pattern to total CBDP and THCP concentrations ($p > 0.05$) respectively. A straightforward picture of total CBD *vs* total CBDP and total THC *vs* total THCP in percentage is illustrated in Fig. 4 (both CBD and THC percentages were calculated out of total C5 cannabinoids, as well as CBDP and THCP results were calculated out of total C7 cannabinoids).

As expected, in THC-predominant plants THC and THCP covered the almost total percentage of total C5 and C7 cannabinoid fractions respectively, while CBD and CBDP represented only a small percentage ($p < 0.001$, Fig. 4). The opposite trend (low THC and THCP and high CBD and CBDP content) was observed in CBD-rich plants belonging to chemotype III ($p < 0.001$). No significant difference was observed in the percentages of total THCP/C7 compared to total THC/C5, as well as between total CBDP/C7 and total CBD/C5 in chemotype III plants ($p > 0.05$), suggesting that the distribution of the C7 phytocannabinoids follows that of their C5 homologs, thus being representative of the belonging chemotype. Only chemotype IV presented significant differences between the investigated couples of phytocannabinoids ($p < 0.001$), most likely due to the very low if not null concentrations of C7

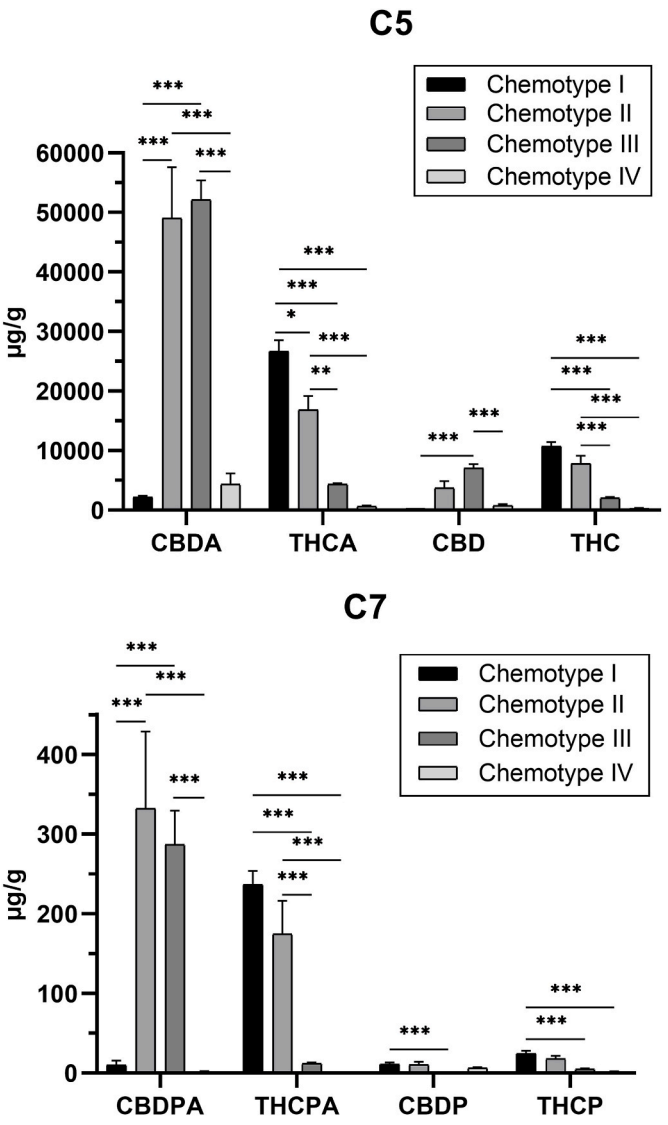


Fig. 3. Distribution of C5 and C7 CBD-type phytocannabinoids among chemotypes I-IV. Average amount of CBDA, CBD, THCA, and THC (C5) in the four chemotypes (I, II, III, and IV) calculated as μg/g and reported as mean ± SEM ($n = 3$) (top). Average amount of CBDPA, CBDP, THCPA, and THCP (C7) in the four chemotypes (I, II, III, and IV) calculated as μg/g and reported as mean ± SEM ($n = 3$) (bottom). Significant difference is expressed through asterisks: *p < 0.05, **p < 0.01, ***p < 0.001.

species in this chemotype ($p < 0.05$). Compared to their C5 counterpart, C7 homologs are consistently less abundant in all accessions, thus suggesting that the final concentrations of these new phytocannabinoids are generated from less abundant substrates. Considering that CBGA is the substrates used by CBDA- and THCA synthase to produce CBD-type and THC-type compounds respectively, the concentrations of C7 phytocannabinoids should be strictly connected to those of CBGPA and its precursors [20] and to a different affinity and catalytic activity of THCAS and CBDAS towards alkyl homologs [21]. As a result, C7 cannabinoids represented only a small percentage out of the sum of C5 and C7 cannabinoids ($p < 0.001$) ranging from 0.11% to 1.27%, which might reflect the low abundance of the substrate CBGPA (and its phorolic precursors).

The experimental data indicated that almost all the samples tested presented a variable amount of the novel C7 phytocannabinoids, whose pharmacology is still unexplored. From preliminary results, Δ^9-THCP showed an extraordinary activity profile with 33-fold *in vitro* affinity for

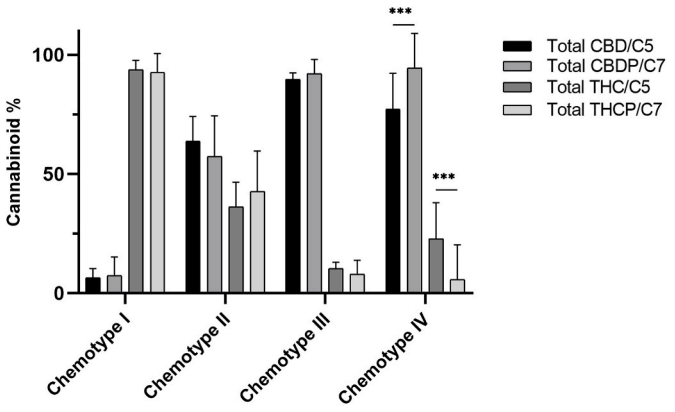


Fig. 4. Distribution of total CBD-type and total THC-type phytocannabinoids among chemotypes I-IV. Percentages of total CBDP (calculated as the sum of (CBDPA*0.877) and CBDP out of C7 phytocannabinoids), total CBD (calculated as the sum of (CBDA*0.877) and CBD out of C5 phytocannabinoids), total THCP ((THCPA*0.877+THCP)/C7), and total THC ((THCA*0.877)+Δ^9-THC/C5) in the four chemotypes (I, II, III, and IV). Significant difference is expressed through asterisks: *p < 0.05, **p < 0.01, ***p < 0.001.

CB_1 receptors compared to its C5 homolog and a cannabimimetic behavior similar to Δ^9-THC but at lower doses [7]. Although Δ^9-THCP concentrations here reported were in the order of μg/g, a potential therapeutic effect should not be completely ruled out. In this regard, fiber-type and drug-type cannabis can be distinguished by a THC cut-off of 0.2%, above which the plant is classified as drug-type. The highest level of THCP was registered for CINRO at about 0.5 mg/g (0.05% on inflorescence dry weight). Considering the higher biological activity compared to THC and the complexity of its pharmacokinetics in the human body, such potency could hypothetically be equivalent to a plant with a THC content higher than 1%.

Moreover, it should be taken into account that thanks to the new frontiers in cannabis chemotype breeding it is possible to produce high potency plants with increased levels of these novel phytocannabinoids. On the other hand, although CBDP pharmacological role has not been cleared to date, plants rich in this phytocannabinoid could be potentially bred for the treatment of important inflammatory pathologies.

4. Conclusions

The identification of the novel C7 phytocannabinoids, THCP and CBDP, has given the opportunity to study their distribution in cannabis germplasm. In order to provide a reliable estimate of their amount in fresh cannabis plants it is necessary to quantify the native acidic species of phorolic cannabinoids. A stereoselective synthesis of these compounds has allowed for the first time to obtain the analytical standards for a semi-quantitative determination. By employing the UHPLC-HRMS method based on a targeted approach towards both C5 and C7 acidic and neutral phytocannabinoids, it was possible to highlight a heterogeneous distribution of such compounds among forty-nine samples with different chemotypes. Although they represented only a small percentage out of total C5 and C7 species, it should be taken into account that the C7 THC homolog resulted more active than THC itself. Therefore, the concentration of the C7 species observed in the present study for some accessions could be considered relevant for therapeutic purposes. In particular, C7 phytocannabinoids showed a trend of concentrations strictly depending on the chemotype, with high THCP concentrations in chemotype I accessions (almost pure in THC) and high CBDP concentrations in chemotype III plants (almost pure in CBD). As a result, a future direction of cannabis research may focus on the screening and selection of favourable genetics with high content of C7 phytocannabinoids to be employed in the treatment of specific pathologies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2021.122704.

Author disclosure statement

No competing financial interests exist.

Credit author statement

C. Citti, P. Linciano: Supervision, Investigation, Writing – original draft; C. Citti, F. Tolomeo, L. Carbone: (UHPLC-HRMS) Investigation, Formal analysis; P. Linciano, F. Russo, G. Biagini: (Synthesis) Investigation; G. Biagini: (Statistical analysis) Formal analysis; R. Paris, F. Fulvio, N. Pecchioni: Resources; Writing – original draft; A. Laganà, A. L. Capriotti, M. A. Vandelli: Data curation; G. Gigli: Resources; G. Cannazza: Conceptualization, Funding acquisition, Project administration; all authors: Writing – review & editing.

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