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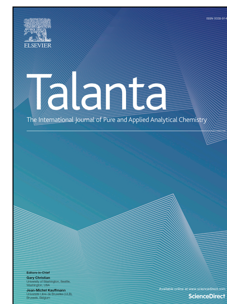

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CRediT author statement

Carmela Maria Montone: Methodology; **Andrea Cerrato:** Data Analysis; **Bruno Botta:** Supervision; **Giuseppe Cannazza:** Project Administration; **Anna Laura Capriotti:** Supervision **Chiara Cavaliere:** Writing – Original Draft; **Cinzia Citti:** Investigation, Writing - Review & Editing; **Francesca Ghirga:** Investigation; **Susy Piovesana:** Writing - Review & Editing; **Aldo Laganà:** Supervision, Project Administration

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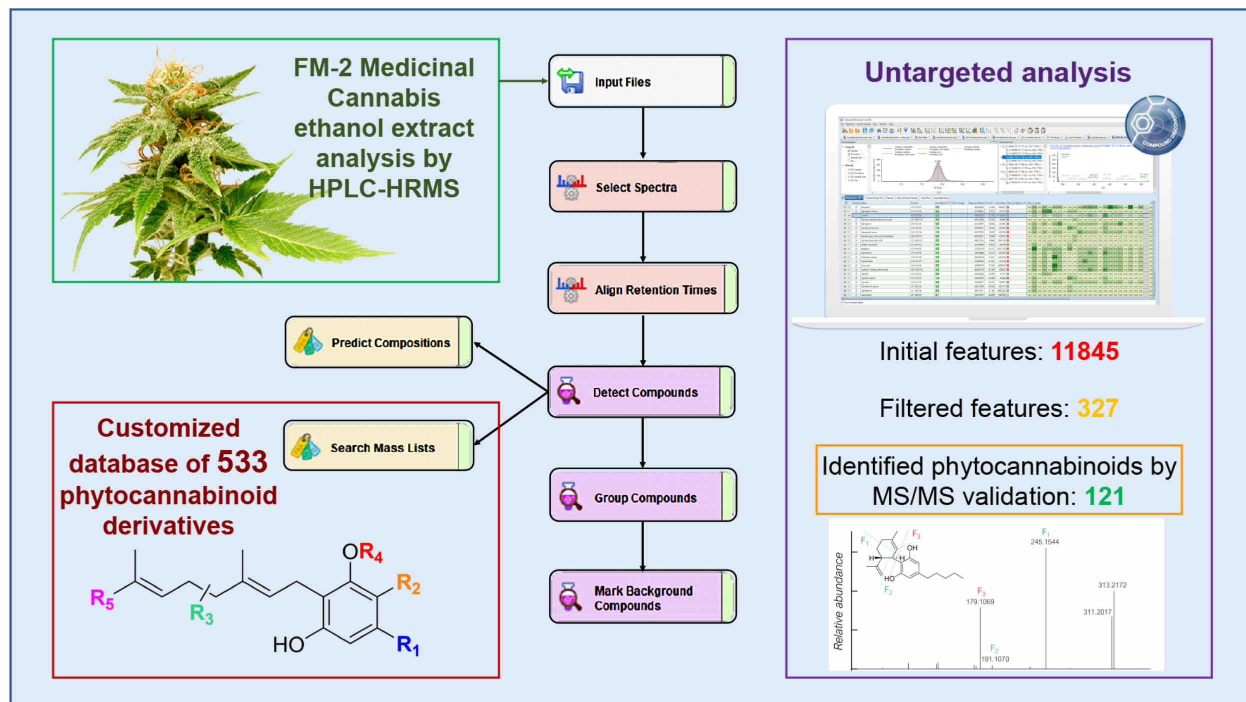

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Improved identification of phytocannabinoids using a dedicated structure-based workflow

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Abstract

Phytocannabinoids are a broad class of compounds uniquely synthesized by the various strains of *Cannabis sativa*. Up to date, most investigation on phytocannabinoids have been addressed to the most abundant species, Δ^9-tetrahydrocannabinol and cannabidiol, for their well-known wide range of pharmaceutical activities. However, in the recent years a large number of minor constituents have been reported, whose role in cannabis pharmacological effects is of current scientific interest. With the purpose of gaining knowledge on major and minor species and furnishing a strategy for their untargeted analysis, in this study we present an innovative approach for comprehensively identifying phytocannabinoids based on high-resolution mass spectrometry in negative ion mode, which allows discrimination of the various isomeric species. For a faster and more reliable manual validation of the tandem mass spectra of known and still unknown species, an extensive database of phytocannabinoid derivatives was compiled and implemented on Compound Discoverer software for the setup of a dedicated data analysis tool. The method was applied to extracts of the Italian FM-2 medicinal cannabis, resulting in the identification of 121 phytocannabinoids, which is the highest number ever reported in a single analysis. Among those, many known and still unknown unconventional phytocannabinoids have been tentatively identified, another piece in the puzzle of unravelling the many uncharted applications of this matrix.

Keywords

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Cannabis sativa; phytocannabinoids; untargeted analysis; Compound Discoverer; high-resolution mass spectrometry

Abbreviations

HRMS: high-resolution mass spectrometry

CBD: cannabidiol

THC: tetrahydrocannabinol

CBG: cannabigerol

CBC: cannabichromene

CBL: cannabicyclol

CBN: cannabinol

CBDN: cannabidinol

CBE: cannabielsoin

CBT: cannabitriol

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1. Introduction

Phytocannabinoids are a class of terpenophenolic compounds uniquely found in the various strains of *Cannabis*[1]. Despite being employed for thousands of years and already introduced in the western world in the nineteenth century for its analgesic, anti-inflammatory and narcotic properties[2], cannabis psychoactive effects had induced most governments, up to recently, to list it as Schedule I drug, implying, in fact, no legal uses for medicinal applications. In the recent

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years, however, investigations in neurobiology of cannabis assumption led to the discovery of the endocannabinoid system[3,4], generating an ever increasing interest in the pharmaceutical and therapeutic fields, which has nowadays prompted a partial lifting of the restrictions in the usage of medicinal Cannabis. Δ^9-tetrahydrocannabinol (Δ^9-THC), the most abundant and notorious phytocannabinoid, has been largely investigated for its ability to bind cannabinoid receptors CB_1 and CB_2 with a strong binding affinity, causing the well-known intoxicating psychoactive effect[5]. Other phytocannabinoids, e.g. cannabidiol (CBD), have been proven to interact with non-CB molecular targets, such as glycine receptors[6], G protein-coupled receptors[7] and serotonin receptors[8]. THC is responsible for partial agonist activity to both CB receptors and enacts a wide range of pharmacological effects, such as appetite stimulation and relief of neuropathic pain in patients suffering from multiple sclerosis[5,9], while CBD has much lower binding affinity and displays antagonist activity instead, tempering the psychoactive effects of THC[10]. Moreover, CBD interacts with several non-cannabinoid receptors, carrying out various neuroprotective functions, ranging from reducing oxidative stress to anxiolytic, antidepressant, anticonvulsant and antiarthritic activities[11]. On the basis of their Δ^9-THC and CBD content, the numerous strains of *Cannabis sativa* are usually distinguished into drug-type (high THC content) and fiber-type (high CBD content)[12].

In addition to the two aforementioned compounds, more than one hundred other phytocannabinoids have been identified to date[13], divided into 11 subclasses according to their structure: cannabigerol (CBG)-type, Δ^9-THC-type, CBD-type, cannabichromene (CBC)-type, cannabinol (CBN)-type, Δ^8-tetrahydrocannabinol (Δ^8-THC)-type, cannabicyclol (CBL)-type, cannabidinol (CBND)-type, cannabielsoin (CBE)-type, cannabitriol (CBT)-type and miscellaneous type[14]. Those species are synthesized in the glandular trichomes of the female

flowering and fruiting tops in their acidic form, and eventually undergo spontaneous non-enzymatic decarboxylation, generating the more familiar neutral species[12,13]. As a matter of fact, it is worth mentioning that several classes of phytocannabinoids, like CBT-like and CBE-like, are likely oxidation products generated either in the plant or during storage, drying and extraction phases.

Up to now, most studies profiling phytocannabinoids report only the major constituents[15–19]. whereas it has been demonstrated that cannabis extracts from different strains showed contrasting anti-convulsant effects despite possessing equally high CBD concentrations, meaning that low-abundance cannabinoids could play a crucial role in determining the pharmaceutical properties of cannabis and its derivatives[20]. With the purpose of gaining knowledge on less notorious compounds, high-performance liquid chromatography (HPLC) coupled to high-resolution mass spectrometry (HRMS) is the foremost technique for the comprehensive characterization of phytocannabinoids[20–23]. Even though *n*-propyl homologues of THC and CBD have been known for a long time[5]. more recently, other unorthodox homologues of Δ^9-THC and CBD presenting alkyl-resorcinyl chains of varying length have been isolated and characterized for the first time by means of HRMS analysis[24–26]. In particular, one of Δ^9-THC homologues, named Δ^9-tetrahydrocannabiphorol (Δ^9-THCP) since presents a heptyl-resorcinyl moiety, was isolated from the Italian FM2 medicinal cannabis variety and showed a cannabimimetic activity several times higher than its common pentyl homologue[26].

In this study we present an innovative approach for comprehensively identifying phytocannabinoids based on HRMS data. For a faster and more reliable manual validation of MS/MS spectra of known and still unknown species, an extensive database of phytocannabinoid derivatives was compiled and implemented on Compound Discoverer software (Thermo Fisher

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Scientific) for the automatic match of extracted features to those present in the database. A detailed study of cannabinoid fragmentation pathways was achieved for the correct identification of the extracted compounds. The aim of the work is creating a workflow specifically dedicated to a faster and more exhaustive analysis of phytocannabinoids, which conjugates acquisition in untargeted fashion to suspect screening data analysis. The method also enables the potential discovery of still uncharted species for a better understanding of cannabis chemical composition and further uses in pharmaceutical and therapeutic fields. To the best of our knowledge, this is the first example of a dedicated data processing workflow for the simultaneous identification of all phytocannabinoid derivatives. The method was applied to extracts of the Italian FM-2 medicinal cannabis, variety which was chosen for its balanced content of both THC and CBD as well as other non-canonical pytocannabinoids[25,26].

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2. Materials and methods

2.1. Chemicals and Materials

Ethanol 96% (analytical grade), acetonitrile, water and formic acid (LC-MS grade) were purchased from Carlo Erba (Milan, Italy). Δ^9-Tetrahydrocannabivarin (Δ^9-THCV), Δ^9-THC, cannabidivarin (CBDV), CBD, CBG, CBN, CBC, cannabigerolic acid (CBGA),

tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA) were purchased as Cerilliant certified analytical standards (Sigma-Aldrich, Milan, Italy).

2.2. Cannabinoid extraction and HPLC-HRMS analysis

FM-2 inflorescence (batch n. 6A32/1) was supplied by the Military Chemical Pharmaceutical Institute (Florence, Italy) and used for extraction and analysis by the Department of Life Sciences of the University of Modena and Reggio Emilia with the authorization of the Italian Ministry of Health (prot. n. SP/062). The raw material (1.5 g) was finely ground with a coffee grinder and divided into two batches. One batch (500 mg) was extracted without further treatment (native FM2), whereas the other one (1 g) was placed in an oven at 120 °C for 2 h to achieve decarboxylation (decarboxylated FM2). After cooling to room temperature, 500 mg were extracted as indicated by the monograph of *Cannabis flos* reported in the German Pharmacopoeia[27]. Briefly, FM2 inflorescence, either in native or decarboxylated form, was suspended in 20 mL of 96% ethanol and stirred at room temperature for 15 min. The extract was transferred into a volumetric flask and the solid residue further extracted with 12.5 mL of 96% ethanol. This step was repeated with further 12.5 mL of 96% ethanol and the volumetric flask was filled up to 50 mL with fresh ethanol. A 1 mL aliquot was filtered through a 0.45 µm cellulose membrane filter and diluted (×100) with acetonitrile. This solution (5 µL) was injected into a Thermo Fisher Scientific Ultimate 3000 liquid chromatograph (HPLC), which is equipped with a vacuum degasser, a binary pump, a thermostated autosampler, and a thermostated column compartment. The chromatographic separation was carried out on a core shell C_{18} stationary phase (Poroshell 120 SB-C18, 3.0 × 100 mm, 2.7 µm, Agilent, Milan, Italy) eluting a mobile

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phase of 0.1% aqueous formic acid (A) and acetonitrile (B). A linear gradient from 5% to 95% B was set over 20 min, followed by an isocratic elution at 95% B for 5 min, and re-equilibration to the initial conditions for 5 min. The flow rate was maintained at 0.5 mL/min throughout the run. The chromatographic section is interfaced to a heated electrospray ionization source (HESI) with the following settings: 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. The analyzer consisted of an Orbitrap HR mass spectrometer operating in full scan data-dependent acquisition (FS-dd-MS^2) in positive and negative mode at a resolving power of 70,000 full width at half maximum (FWHM) @*m/z* 200. After optimization by direct infusion of a mixture (5 μg L^{-1}) of the available cannabinoid standards, the parameters of the Orbitrap mass analyzer were set as follows: scan range, *m/z* 150-750; AGC, 3e6; injection time, 100 ms; isolation window, *m/z* 0.7. The collision energy for the fragmentation of the molecular ions was set at 20 eV. The analyses were performed using Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA).

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2.3. Phytocannabinoid database compilation

The database of phytocannabinoids was generated by using Excel in consideration of the structural variability of the major and minor species identified to date[20,21,28]. Eleven classes of cannabinoids were considered: CBG-type, Δ^9-THC-type, CBD-type, CBC-type, CBN-type, Δ^8-THC-type, CBL-type, CBND-type, CBE-type, CBT-type and miscellaneous type[14]. As regards miscellaneous-type, several species were listed: cannabiripsol (CBR)[29], cannabicitran (CBCT)[30], cannabitetrol (CBTT)[31], cannabifuran (CBF)[32], dehydrocannabifuran

(DCBF)[32], cannabimovone[33], and cannabichromanone[34]. All species were inserted both in the neutral and in the acidic forms. For all listed classes, alkyl-resorcinyl moieties from one to ten carbon atoms were considered, together with hydroxyl and methyl derivatives. Homologues of CBG-type compounds with longer prenyl moieties were also added to the database[35]. After generating the combinations, a list 533 cannabinoid derivatives was obtained and implemented on Compound Discoverer 3.1 (Thermo Fisher Scientific).

2.4. Data analysis and cannabinoid identification

For both native and decarboxylated sample, raw data from three experimental replicates and a blank sample were processed using a workflow designed as follows (Figure S1). The customized database compiled in section "Phytocannabinoid database compilation", and complete of IDs, masses and molecular formulas, was implemented in *mass lists* feature for the automatic matching of extracted *m/z* ratios to compounds present in the database. Moreover, parameters for predict composition were adapted to the analysis of phytocannabinoids. The *minimum element counts* was set at $C_{15}H_{15}O$, while the maximum at $C_{35}H_{60}O_{10}$, in order to automatically reject species possessing molecular formulas which could not correspond to those of cannabinoids. The complete set of parameters inserted in the dedicated data analysis workflow is available in Table S1. Extracted masses from the chromatograms were aligned and filtered to remove background compounds present in the blank sample, features whose masses were not present in the databases and those which were not fragmented. Finally, MS/MS spectra of the filtered features were manually validated to assign the tentative identification according to the typical fragmentation pathways of the eleven classes of compounds[20]. The nomenclature of identified cannabinoids

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was given according to the literature and has been reported in *Supplementary Material*. Data for the tentatively identified compounds are summarized in Table S2 with the related confidence level according to Schymanski *et al*[36]. In particular, level 1 refers to compound identified by match of exact mass, MS/MS spectrum and retention time (r_T) to those of available standards, level 2a refers to compounds tentatively identified by matching MS/MS spectra to the literature data or online spectral databases, whereas level 2b refers to those tentatively identified by study of diagnostic fragments in MS/MS spectra but not supported by data available in the literature.

3. Results and discussion

3.1. Customized workflow on Compound Discoverer

Δ^9-THC and CBD, along with a few other cannabinoids, have been extensively investigated by targeted MS for obtaining quantitative results, which are particularly important in the case of *Cannabis sativa.* Based on their relative content, different strains of cannabis are in fact classified as fiber-type or drug-type[12], often also determining its legal status. In this regards, extraction procedures[37], as well as separation methods[38,39], have been widely studied. However, the more the interest in cannabis has increased for pharmaceutical and therapeutic applications, the more the knowledge on hemp phytocannabinoid content has grown in recent years, resulting in the need for a different approach rather than targeted analysis. Whether comprehensive characterization of strains of cannabis are needed, low-abundance compounds are searched or metabolites of cannabinoids after assumption are investigated, untargeted analyses represent the most viable strategy. Untargeted approaches based on HPLC coupled to HRMS

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permit the simultaneous collection of large sets of data of both known and unknown compounds while forgoing the opportunity to perform quantitative analysis[40]. Moreover, targeted analyses furnish extremely rapid and straightforward results, while data analysis of the gigantic sets of data collected in untargeted fashion cannot be carried out manually, and dedicated software programs for the extractions of features from raw data are generally required[41]. Thanks to MS-based data processing software programs, *m/z* ratios and their associated MS/MS spectra are extracted and aligned, and diverse adducts deriving from the same compound are grouped, thus generating a list of features to manually validate according to r_T, masses and diagnostic product ions.

Furthermore, with the purpose of streamlining the manual validation, data processing programs grant the access online MS-based databases and libraries for automatic matches of features to compound names, structures and, sometimes, recorded MS/MS spectra. Even the most complete available databases, however, do not possess exhaustive data for structure-related classes of compounds, such as phytocannabinoids, especially when it comes to the study of unreported or unknown species. Moreover, since small molecule masses and molecular formulas are shared among many diverse species, broad range database are often unsuitable for the profiling of a specific class of compounds. Therefore, a different approach for raw data analysis was chosen. By means of Excel, a database of reported and unreported cannabinoid derivatives was compiled, according to the structural modifications reported in the literature[20,21,28]. The ten main classes of phytocannabinoids possess rather consistent structures, comprised of an alkyl-resorcinyl portion bound to a prenyl moiety, whose structural diversity determines the partition in classes. The typical alkyl chain is made up of five carbon atoms, constituting a pentyl-resorcinyl moiety that is common to all classes, as in the case of THC and CBD (Figure 1).

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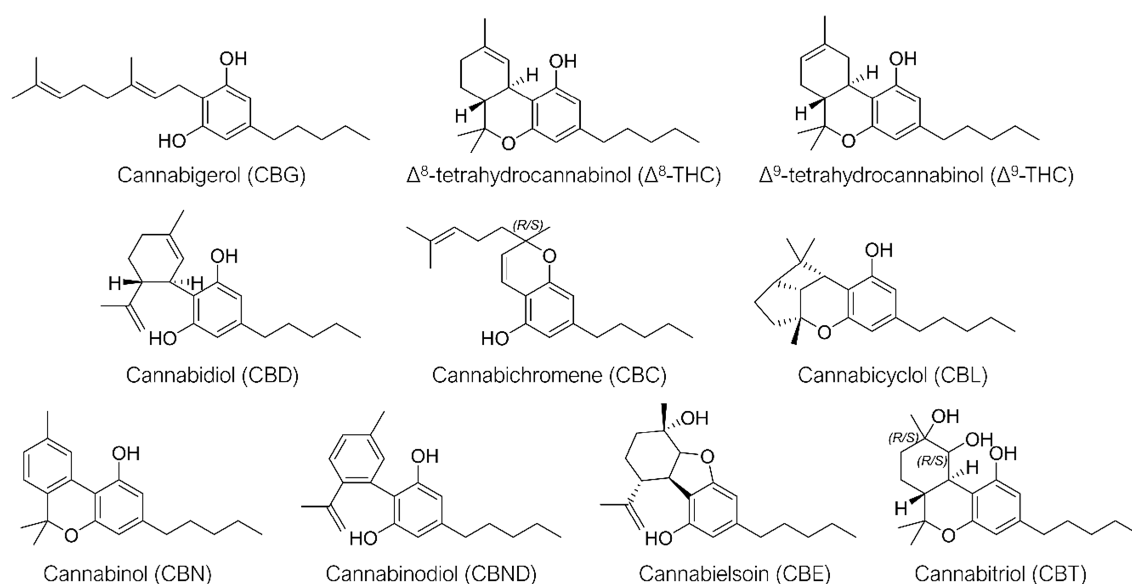

Figure 1. Structures of the archetypes of the ten main classes of cannabinoids.

Most cannabinoids possessing unorthodox structures, such as CBR or CBF, are grouped into the eleventh class under the name of miscellaneous-type phytocannabinoids. All compounds exist, and were therefore listed, in a native acid form and the more common neutral form. Despite being considerably less abundant, several methyl, propyl, butyl and heptyl homologues of THC or CBD have been recently isolated and characterized[5,24–26,42]. With the purpose of exploring known and unknown alkyl chain analogues of cannabinoids, one to ten carbon atom chains homologues of all classes, both in neutral and in acid forms, were inserted. Moreover, since some hydroxylated derivatives of THC and CBD have been reported both in plant matrix and after human assumption[43,44], hydroxyl derivatives of all the series of homologues were generated. *O*-methylated cannabinoids were also considered, as well as CBG-type compounds possessing farnesyl moieties rather than most common prenyl chains were included[28]. Finally

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a database of 533 cannabinoid derivatives was obtained and implemented on Compound Discoverer as a mass list (Figure 2).

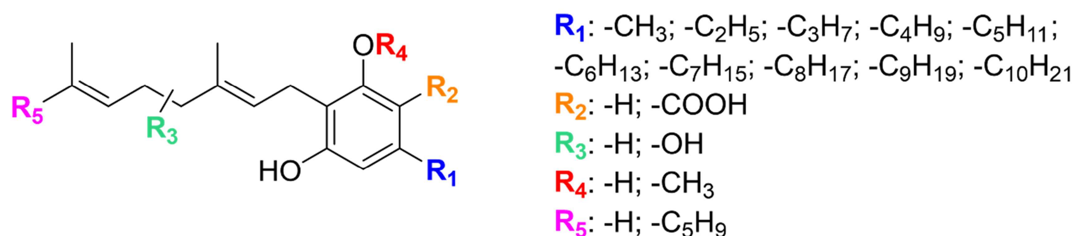

Figure 2. Structural modifications considered for phytocannabinoid database compilation.

For cannabinoid software-assisted identification, a dedicated workflow was set up (Figure S1). Parameters for *predict composition* tool were adapted to the compounds present in the database, with the purpose of filtering all compounds possessing molecular formulas that were incompatible to those of listed species. *Mass lists* tool executes the automatic matches of features to compounds present in the database based on matches and molecular formulas, regardless of the polarity of the mass-spectrometric acquisition or the adducts generated in the ESI source. Before the manual validation of putative compounds, several filters were applied to remove most false positives, resulting in a drastic decrease of the number of features (less than 3% of the original amount) that led to a decisive streamlining of MS/MS spectra manual validation.

3.2. Phytocannabinoid identification

To test the potential of the developed method, the mix of standard cannabinoids analyzed both in positive and in negative ion polarity was processed by Compound Discoverer, since studies on

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cannabinoids have been carried out in either polarities[16,20]. Our ten available authentic standards were all correctly identified, however ionization efficiencies present opposite trends. Neutral species were more efficiently charged in positive ion mode, while, not unexpectedly, negative polarity performed better for carboxylated species. Although positive polarity performs overall better in terms of ionization efficiency, other issues shall be considered when untargeted analyses are arranged. Several classes of phytocannabinoids present in fact the same elemental composition, therefore, MS/MS spectra shall be distinguishable, since m/z ratios cannot permit any discrimination. In Figure 3, spectra of standard CBD, THC and CBC both in positive and in negative polarity are shown. MS/MS spectra of the three isomers are rather complicated and almost indistinguishable in positive mode, implying that identification can be achieved only by matching r_T if authentic standards are available. Spectra recorded in negative polarity, instead, are much clearer and discernible, with fragmentations sequentially occurring on the prenyl moiety (F_1, F_2 and F_3). CBD (Figure 3a) presents high abundance F_1 (*m/z* 245.1544) and F_3 (*m/z* 179.1069) product ions and an intense peak at *m/z* 311.2017 (loss of H_2), while THC (Figure 3b) presents a rather scarce fragmentation thanks to its more stable structure. Finally, CBC (Figure 3c) produces a peculiar F_1 ion (*m/z* 243.1392) due to the diverse cyclization of the prenyl moiety and an intense *F2* ion (*m/z* 191.1070). Since fragmentation pathways of cannabinoids involve mainly the prenyl moiety, the identification of cannabinoids possessing modification of the alkyl-resorcinyl section is straightforward[20,26,45]. Based on these considerations, negative polarity was considered the best choice for untargeted identification of phytocannabinoids based on the well-known fragmentation pathways[20].

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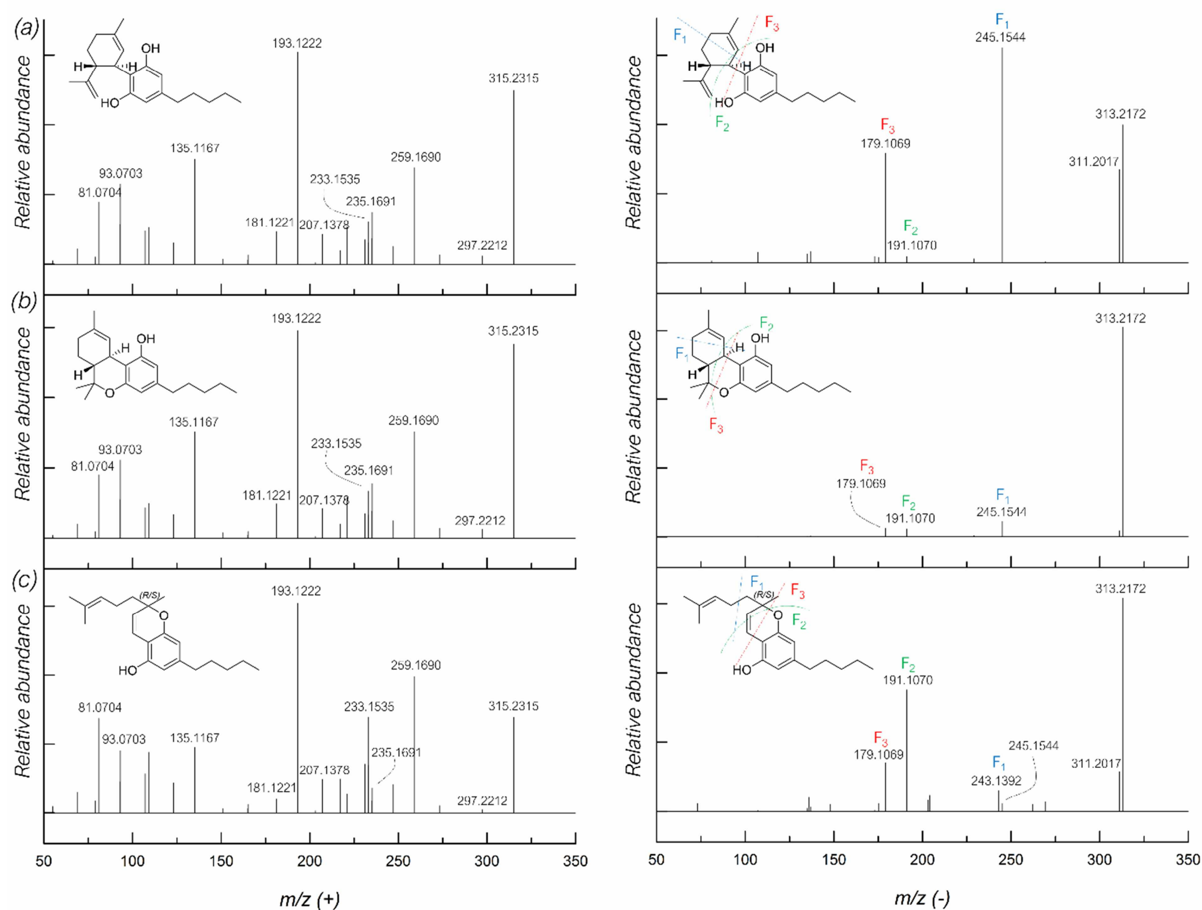

Figure 3. MS/MS spectra of standard CBD *(a)*, THC *(b)* and CBC *(c)* in positive and negative polarity. Diagnostic fragments for cannabinoid identification in negative polarity are marked as F_1, F_2 and F_3.

By studying the product ions deriving from prenyl fragmentation, a large number of alkyl homologues of the various classes of phytocannabinoid was eventually identified. Their validation was supported by r_T, which gradually increases for compounds belonging to the same class along with the elongation of the alkyl chains. As regards Δ^8-THC-type species, these compounds usually elute at longer r_T than the more common Δ^9-THC-type isomers[20,21]. Due to the typical fragmentation pathways of THC-type compounds, the two isomers present the

same fragmentation spectrum[20]. Therefore, identified THC-type compounds were not labeled; however, since there are no peaks possessing the same *m/z* and MS/MS spectrum at longer r_T than supposedly high-abundance Δ^9-THC-type compounds, it is legitimate to assume that no Δ^8-THC-type compounds were identified in FM-2 cannabis.

Compound 37 was identified as demethylated derivative of CBDA; since it presented the same F_1, F_2 and F_3 as regular CBDA, the demethylation could have occurred at position *7* as a result of microbial oxidation[46]. Compounds 46 and 47 have been identified as hydroxylated derivatives of CBD; also in these cases, the three main fragments corresponded to those of CBD and ions deriving from the loss of CH_2O and CH_4O could indicate the presence of a primary alcohol. Similarly, compound 88 was identified as hydroxyl THC and compound 99 as hydroxyl CBC. Moreover, several hydroxyl derivatives of CBN were identified. CBN, whose aromatization of the prenyl moiety causes fragmentation of the resorcinyl section, produces a typical fragment at *m/z* 171.0815 after previous loss of two methyl groups (*m/z* 279.1930); hydroxylated derivatives generate, instead, an analogous fragment at *m/z* 187.0765, which indicates hydroxylation on the aromatized prenyl moiety. Compound 108 presents peaks compatible with a homologue of CBDA with an extra CH_2; considering the presence of a F_3-CH_3 ion at m/z 178.0991 and the much higher r_T compared to the series of CBD-type, it was identified as *O*-methyl CBDA. Compound 56 was identified as cannabifuranic acid (CBFA) by comparison with its isomer CBNA; while the latter undergoes loss of two methyl groups (30 Da), CBFA loses the isopropyl moiety (42 Da) before generating the fragment at *m/z* 171.0815. Compound 73 was identified as dehydrocannabifuranic acid (DCBFA) with the same logic. Finally, CBR-type cannabinoids, which are dihydroxylated derivatives of THC-type species, present two sequential water losses while sharing fragments F_2 and F_3 with THC[23].

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3.3. FM2 cannabinoid composition

By means of Compound Discoverer, 121 cannabinoids were tentatively identified in the Italian FM-2 medicinal cannabis variety, which to our knowledge, is the highest ever reported for a single and simultaneous analysis of cannabis plant extract. In terms of number of identifications, CBE- and CBT-type phytocannabinoids were the most numerous, with 28 and 23 identified compounds respectively, ahead of CBD-, CBN- and THC-type, with 14, 14, and 12 identifications, respectively. Those large number are partly explained by the different stereoisomers that derive from hydration of double bonds. Moreover, such more hydroxylated compounds were believed to be more efficiently ionized by the ESI source in negative polarity. As expected, in terms of peak areas, the most abundant species in native samples were CBDA and THCA, although several non-enzymatic derivatives, such as CBEA, CBTA and CBRA, present significant abundances. In Figure S2, a summary of the total areas per class of cannabinoids is shown. Obviously, since ionization efficiency probably depends on the number of free hydroxyl groups in negative polarity, peak areas cannot be directly associated to real concentrations for different classes of cannabinoids. Among the 10 classes, CBD-type cannabinoids were by far the most abundant with nearly 50% of the total area, followed by CBE-, CBT- and THC-type species with 14%, 12% and 9%, respectively. On the other hand, CBL- and CBND-type cannabinoids were present in minor quantities. It is worth mentioning that about 36% of the total peak area is represented by species which are not native metabolites of cannabis, but are produced by non-enzymatic reactions mainly during harvest and storage[1]. Several cannabinoids presenting non-pentyl alkyl chains were identified, e.g. an ethyl homologue of

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THCA, a cannabinoid presenting a two carbon alkyl chain which was never reported before. Compound 83, in fact, presents *m/z* and product ions which differ from those of THCOA by 14.0157, which is unequivocally due to an extra CH_2. Since all major product ions present the same difference, the extra methylene must be on the alkyl-resorcinyl moiety. Moreover, due to its r_T (18.21 min) being between those of THCOA (17.51 min) and THCVA (19.02 min), the methylene was believed to be on the alkyl chain rather than an *O*-methylation, which would have resulted in a much greater contribution in terms of hydrophobicity. Therefore, compound 83 was tentatively identified as THC(C2)A, the ethyl homologue of THCA. With the same logic, hexyl homologues of THCA, CBGA and CBCA were identified for the first time in cannabis extract. Considering a recent study on heptyl chain homologues of THC and CBD, which were found more bioactive than the regular pentyl compounds[26], these longer alkyl chain homologues could be of great pharmacological interest. Furthermore, several alkyl homologues of less studied classes of cannabinoids were identified for the first time, like heptyl analogues of CBGA, CBCA and CBNA and butyl analogues of CBEA. With respect of previous reported results, several isomers of CBD-like, CBE-like and CBT-like compounds have been reported, presenting the same fragmentation patterns. Therefore, for these highly hydroxylated compounds, those isomers could be stereoisomer rather than positional isomers, which would have resulted in dissimilar MS/MS spectra. As a matter of fact, the largest number of unorthodox homologues were those possessing different alkyl chains, while few hydroxylated and *O*-methylated compounds were identified.

4. Conclusions

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Phytocomplex analysis of extracts is of great significance for the broad range of potential applications of *Cannabis sativa*. However, few papers have dealt with the untargeted identification of phytocannabinoids so far, since manual validation of a large number of features is required. The paper describes the development of a data processing workflow by means of Compound Discoverer software for the comprehensive identification of phytocannabinoids. Untargeted analysis was performed in negative polarity, whose resulting MS/MS spectra allow distinguishing the various isomeric compounds, whereas positive polarity, although better in terms of ionization efficiency, was found inappropriate for this purpose. The proposed analytical workflow is based on a customized database of phytocannabinoid derivatives of the main eleven classes and allows automatic match of feature to the listed compounds, resulting in a drastic streamlining of the manual validation. Thanks to the faster and easier manual validation of the filtered features, together with the aforementioned database, a simultaneous identification of a broad range of major and minor cannabinoids was achieved. This method appears promising for the identification of novel bioactive compounds from such rich matrix, as well as comparative studies of different samples, for a better understanding of the most bioactive strains of *Cannabis sativa* or the finest pedoclimatic conditions for its cultivation.

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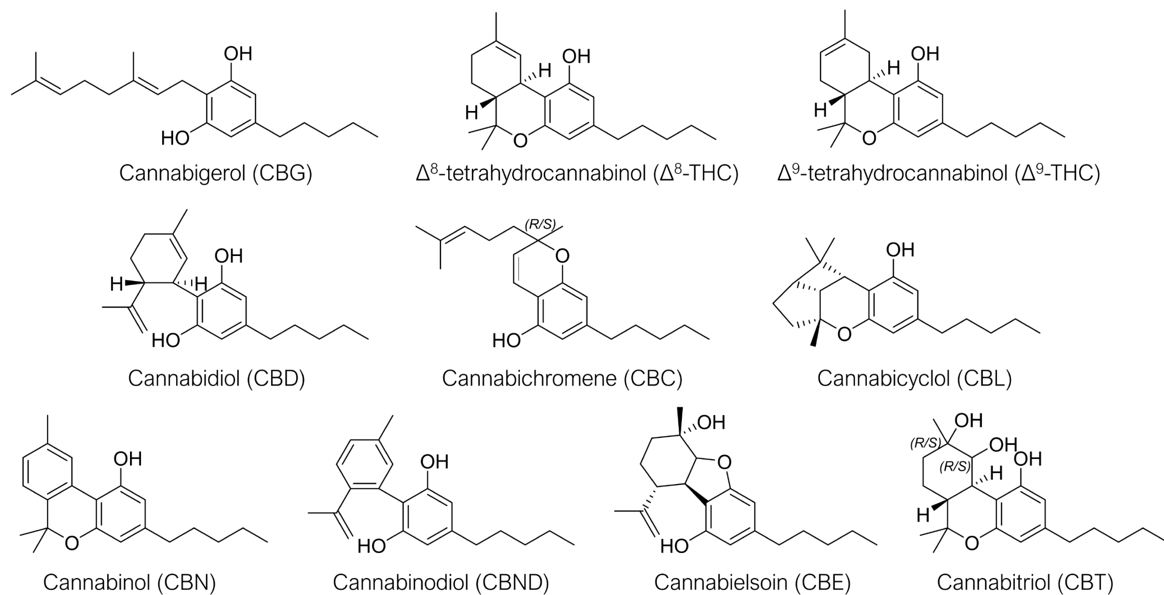

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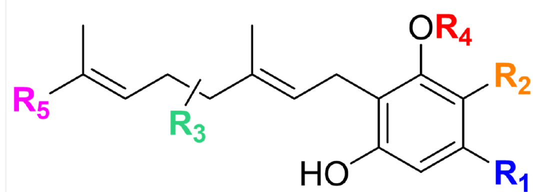

R_1: $-CH_3$; $-C_2H_5$; $-C_3H_7$; $-C_4H_9$; $-C_5H_{11}$; $-C_6H_{13}$; $-C_7H_{15}$; $-C_8H_{17}$; $-C_9H_{19}$; $-C_{10}H_{21}$

R_2: -H; -COOH

R_3: -H; -OH

R_4: -H; $-CH_3$

R_5: -H; $-C_5H_9$

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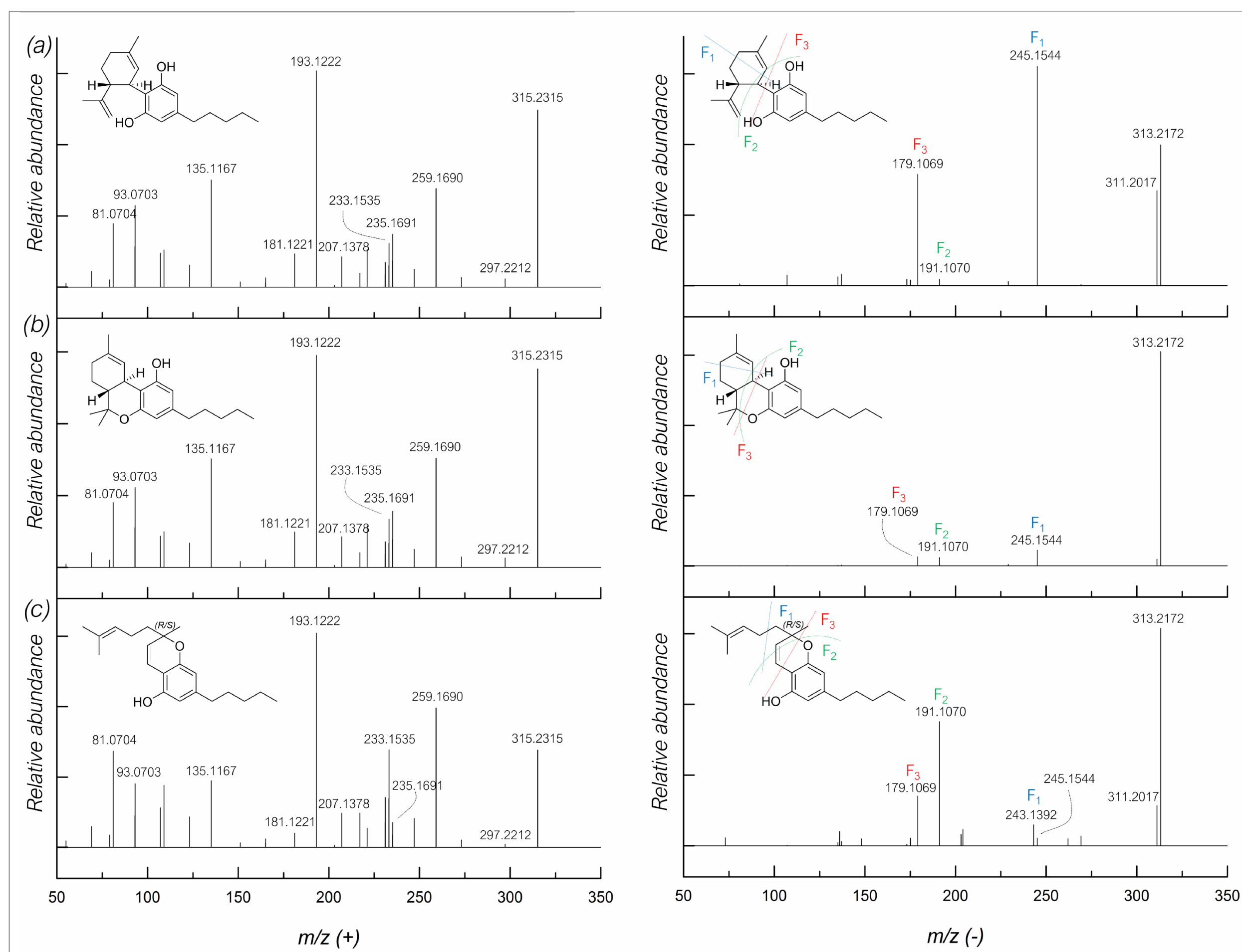
(a)
(b)
(c)
Relative abundance
m/z (+)
m/z (-)
193.1222
315.2315
135.1167
93.0703
81.0704
233.1535
235.1691
259.1690
181.1221
207.1378
297.2212
245.1544
313.2172
311.2017
179.1069
191.1070
243.1392
F1
F2
F3
OH
HO
H
O
(R/S)

Highlights

- A dedicated data analysis method was set up on Compound Discoverer for cannabinoid identification
- A customized database of 533 cannabinoid derivatives was compiled
- Negative ion mode must be operated for distinguishing cannabinoid isomers
- 121 phytocannabinoids were simultaneously identified in FM-2 medicinal cannabis
- Untargeted approaches allow deeper knowledge on cannabinoid composition

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Declaration of interests

☒ 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.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

None

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