Δ^9-Tetrahydrocannabiphorol: Identification and quantification in recreational products

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ABSTRACT

Δ^9-Tetrahydrocannabinol (Δ^9-THC) is known to be the component of the cannabis plant responsible for the psychoactive effects generated by the activation of the endocannabinoid receptor 1 (CBR1). Following extensive structure–activity relationship (SAR) studies on Δ^9-THC, new molecules with increased CBR1 affinity were designed and synthesized over the last decades. The knowledge arising from the pharmacological and synthetic investigations has been extensively used in the recent past by the industry of substances for recreational use also thanks to the 2018 Farm Bill Act in the USA and the incentive for low-THC cannabis (hemp) cultivation in Europe, which have boosted the availability of hemp derived precursors.

As a result, new semi-synthetic natural and pseudo natural cannabinoids related to the most famous Δ^9-THC and often not subjected to legal restrictions are now available in the online market in a broad array of retail products with no preventive study on their pharmacodynamics and pharmacokinetics.

Some of these products (gummies, cannabis flower and a vape cartridge), all declared to contain the most potent among all the known cannabinoids, Δ^9-Tetrahydrocannabiphorol (Δ^9-THCP), were bought from an online shop and tested through LC-HRMS to determine the effective amount of Δ^9-THCP and of other cannabinoids.

All the three samples were found to contain Δ^9-THCP in amounts significantly different from those declared by the producer. Moreover, the application of an untargeted metabolomics approach (cannabinomics) enabled the identification of other cannabinoids including the emerging semi-synthetic hexahydrocannabinol (HHC) and tetrahydrocannabidiol (H4-CBD) together with byproducts of synthetic origin.

Introduction

Δ^9-Tetrahydrocannabinol (Δ^9-THC) (Fig. 1) is the primary psycho-active component of cannabis (*Cannabis sativa* L.) responsible for the "high" effects achieved by stimulating specific receptors in the central nervous system (CNS) called endocannabinoid receptors (CBR). In particular, the cascade of effects produced by the stimulation of the endocannabinoid receptor 1 (CB1R) consists of alteration of neurotransmitter functionality, enzyme activity, prostaglandin synthesis, membrane perturbation, and so forth and it is translated into various effects such as CNS depression, ataxia, psychoactive effects, hypothermia, analgesia, cardiovascular effects, and more [1]. Following its discovery in 1964 by Raphael Mechoulam [2], the pharmacological behaviour of Δ^9-THC has increasingly attracted the attention of researchers and industries, but also of recreational drug users seeking the psychoactive effects ascribed to this compound.

Extensive structure–activity relationship (SAR) studies on the THC molecule were conducted since the early 1970s by Mechoulam and Edery, highlighting the importance of the aliphatic side chain attached to the resorcinol moiety for the affinity to CB1R [3]. Specifically, the side chain of Δ^9-THC is linear and contains five carbon atoms, but it was shown to retain its psychoactive activity with at least three carbon

atoms. Importantly, an increasing number of carbons in the side chain was found to confer higher potency [1].

Besides the numerous synthetic variations of the lead compound with the aim of enhancing the affinity for CB1R and, consequently, the cannabimimetic activity, up to few years ago Δ^9-THC was believed to be the sole naturally occurring molecule to exert the "cannabis-like" effects. Only Δ^8-THC, produced by the thermodynamic degradation of Δ^9-THC, showed similar psychoactive activity. The other two mildly psychoactive compounds known were Δ^9-tetrahydrocannabivarin (Δ^9-THCV) and cannabinol (CBN), the propyl homologue and the oxidation product of Δ^9-THC, respectively (Fig. 1). However, Δ^9-THCV exhibited lower affinity for CB1R compared to Δ^9-THC ($K_i = 75.4$ nM *vs* 39.5 nM) [4], although higher than CBN, which resulted 2–4 times lower compared to that of Δ^9-THC ($K_i = 211.2 \pm 35.0$ nM *vs* 80.3 ± 22.2 nM) [5]. No other molecules with a longer or branched side chain were known in the plant.

In the past five years, three Δ^9-THC homologues with a linear alkyl side chain were discovered in the cannabis plant: Δ^9-tetrahydrocannabutol (Δ^9-THCB) [6], Δ^9-tetrahydrocannabihexol (Δ^9-THCH) [7], and Δ^9-tetrahydrocannabiphorol (Δ^9-THCP) [8], respectively the butyl, hexyl, and heptyl homologue of Δ^9-THC (Fig. 1). Only Δ^9-THCB and Δ^9-THCP were evaluated for their CB1R affinity as Δ^9-THCH was found in very low amount in cannabis. In details, Δ^9-THCB showed an *in vitro* CB1R affinity 3-fold higher than Δ^9-THC ($K_i = 15$ nM *vs* 40 nM) and an *in vivo* cannabimimetic activity similar to that of the latter [6]. Surprisingly, Δ^9-THCP showed a 33-fold higher affinity for CB1R compared to Δ^9-THC ($K_i = 1.2$ nM *vs* 40 nM) and an *in vivo* full-agonist behaviour at the same dose of Δ^9-THC (10 mg/kg) [8].

The discovery of Δ^9-THCP has revolutionized the knowledge around the cannabis chemistry and phytocannabinoid biosynthesis, but has also captured the interest of various industries devoted to the development of recreational cannabis derived products. Notwithstanding Δ^9-THC and Δ^8-THC are Schedule I substances under the 1971 Convention on Psychotropic Substances, Δ^9-THCP has not been examined by the international authorities in these terms and is openly sold as a hemp derived legal product. Indeed, the 2018 Farm Bill Act in the USA has established a clear boundary of legality for all compounds that can be derived from cannabidiol (CBD), which is the main non-psychoactive component of hemp [9]. Under this act, all phytocannabinoids different from Δ^9-THC naturally occurring in hemp, although in trace amounts, are considered legal and can be included in commercial products regardless of their actual concentration. On the other hand, in Europe there is still silence on this matter, thus Δ^9-THCP can be found all over the world in several recreational products.

Marketed THCP products include either Δ^8 or Δ^9 forms, which are probably synthetically produced given their low concentration in cannabis plants [10].

A few years after the discovery of Δ^9-THCP, a cannabinoid claimed to be hemp-derived started to spread in the USA and Europe: hexahydrocannabinol (HHC), the hydrogenated derivative of Δ^8- and Δ^9-THC (Fig. 1) [11,12]. Such cannabinoid is able to provide similar psychoactive effects as Δ^9-THC with the (9R) epimer being the active form and the (9S) the less active as suggested by *in vitro* radioligand assay at CB1R ($K_i = 15$ nM and 176 nM for the (9R) and the (9S) form respectively) and *in vivo* experiments [13–16]. It has been reported that HHC is obtained by semi-synthesis from the conversion of CBD into either Δ^8- or Δ^9-THC and subsequent hydrogenation and that the active epimer (9R) is preferably obtained via the Δ^8-THC intermediate [15,17].

If residual CBD is present during the hydrogenation of THC it can be completely hydrogenated to tetrahydrocannabidiol (H4-CBD) [18]. Otherwise, if the reaction conditions are not suitable to ensure complete hydrogenation, the reaction leads to the formation of partially hydrogenated CBD derivatives. Hence, it is possible to hypothesize that commercial products containing H4-CBD (whether declared or not) may also contain the three forms of H2-CBD as impurities. Ben Shabat et al. observed that incomplete hydrogenation leads to a mixture containing both epimers (at the C3) of the derivative obtained by hydrogenation of the C3-C2 double bond and the isomer obtained by hydrogenation of the C8-C9 double bond, with a marked prevalence of the latter [19]. This latter form of H2-CBD has shown to possess its own pharmacodynamics with interesting properties in the treatment of skin disorders, as well as antibacterial, bactericidal, antioxidant activity and lower toxicity to human skin fibroblasts compared to the parent compound CBD [20,21].

An obvious outcome of the widespread marketing of THCP and HHC is now represented by hexahydrocannabiphorol (HHCP), obtained from the hydrogenation of THCP with the same stereochemical implications as HHC. Very little knowledge has been shared around HHCP and very recently the isolation and identification of the components and stereoisomers has been reported in two types of e-cigarette cartridges
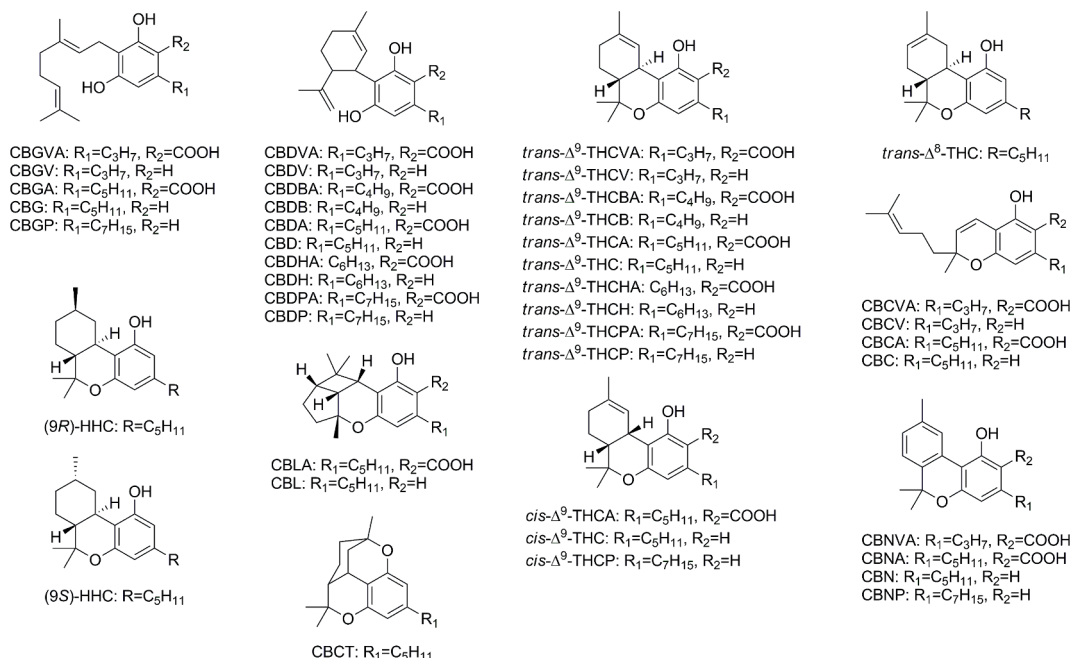

Fig. 1. Core structure of the cannabinoids identified in the recreational products.

CBGVA: R_1=C_3H_7, R_2=COOH
CBGV: R_1=C_3H_7, R_2=H
CBGA: R_1=C_5H_{11}, R_2=COOH
CBG: R_1=C_5H_{11}, R_2=H
CBGP: R_1=C_7H_{15}, R_2=H

CBDVA: R_1=C_3H_7, R_2=COOH
CBDV: R_1=C_3H_7, R_2=H
CBDBA: R_1=C_4H_9, R_2=COOH
CBDB: R_1=C_4H_9, R_2=H
CBDA: R_1=C_5H_{11}, R_2=COOH
CBD: R_1=C_5H_{11}, R_2=H
CBDHA: C_6H_{13}, R_2=COOH
CBDH: R_1=C_6H_{13}, R_2=H
CBDPA: R_1=C_7H_{15}, R_2=COOH
CBDP: R_1=C_7H_{15}, R_2=H

trans-Δ^9-THCVA: R_1=C_3H_7, R_2=COOH
trans-Δ^9-THCV: R_1=C_3H_7, R_2=H
trans-Δ^9-THCBA: R_1=C_4H_9, R_2=COOH
trans-Δ^9-THCB: R_1=C_4H_9, R_2=H
trans-Δ^9-THCA: R_1=C_5H_{11}, R_2=COOH
trans-Δ^9-THC: R_1=C_5H_{11}, R_2=H
trans-Δ^9-THCHA: C_6H_{13}, R_2=COOH
trans-Δ^9-THCH: R_1=C_6H_{13}, R_2=H
trans-Δ^9-THCPA: R_1=C_7H_{15}, R_2=COOH
trans-Δ^9-THCP: R_1=C_7H_{15}, R_2=H

trans-Δ^8-THC: R=C_5H_{11}

CBCVA: R_1=C_3H_7, R_2=COOH
CBCV: R_1=C_3H_7, R_2=H
CBCA: R_1=C_5H_{11}, R_2=COOH
CBC: R_1=C_5H_{11}, R_2=H

(9R)-HHC: R=C_5H_{11}

(9S)-HHC: R=C_5H_{11}

CBLA: R_1=C_5H_{11}, R_2=COOH
CBL: R_1=C_5H_{11}, R_2=H

cis-Δ^9-THCA: R_1=C_5H_{11}, R_2=COOH
cis-Δ^9-THC: R_1=C_5H_{11}, R_2=H
cis-Δ^9-THCP: R_1=C_7H_{15}, R_2=H

CBNVA: R_1=C_3H_7, R_2=COOH
CBNA: R_1=C_5H_{11}, R_2=COOH
CBN: R_1=C_5H_{11}, R_2=H
CBNP: R_1=C_7H_{15}, R_2=H

CBCT: R_1=C_5H_{11}

containing HHC or HHCP using NMR spectroscopy [22]. Moreover, the authors combined three techniques, NMR, GC–MS, and LC-QToF-MS, to identify the unknown components, which included 11α-HHC, 11β-HHC, dihydro-*iso*-THC, 11α-HHCP, and 11β-HHCP [22]. Dihydro-*iso*-THC most likely derives from the hydrogenation of Δ^8-*iso*-THC and $\Delta^{4(8)}$-*iso*-THC, which are obtained during the acid-catalysed cyclization of CBD along with the major products Δ^8- and Δ^9-THC [22,23].

It should be taken into account, however, that each time a synthetic reaction is involved in the production route, safety concerns are raised for the potential contaminants in the final products. Indeed, these contaminants can include not only understudied or unknown cannabinoids but also other reaction side products, raising the possibility of unknown pharmacological and toxicological profiles.

From the analytical point of view, in a perspective of quality control, the presence of all these byproducts from synthetic reactions or of minor cannabinoids from hemp derived raw materials is a challenge because of the lack of the corresponding analytical standards for positive identification using common MS techniques.

To further complicate this scenario pesticides, heavy metals, and excipients are not generally assessed by manufacturers [14]. It should be also noted that most CBD-derived products may contain over the limit Δ^9-THC levels as reaction side products [14,15].

All these new compounds are sold as exotic cannabinoids in a wide range of commercial products including tinctures, distillates, vape cartridges, gummies as well as spiked on cannabis inflorescence [24]. Sometimes these products may be purchased accompanied by a certificate of analysis dealing merely with the concentration of the main active compound or the percentage of Δ^9-THC (for legal requirements) but all the aforementioned contaminants are not reported. Beside this, all both known and unknown cannabinoids can be present at remarkable concentrations with consequent unpredictable side effects. Indeed, unlike the common synthetic cannabinoids, which are known among users to cause serious side effects, these pseudo-natural exotic cannabinoids claim to have the same beneficial effects of cannabis and are masked under the alleged legality of hemp that can be misinterpreted for harmlessness.

The lack of knowledge on the real content of these recreational retail products and of scientific research on the pharmacology of all these new cannabinoids, together with the simultaneous uncontrolled spreading of their recreational use, pose a serious risk for public health and safety. Hence, the purpose of this article is to disseminate what emerged from the analysis of some freely available products purchased online and advertised to contain Δ^9-THCP. Samples of gummy candies, vape cartridge, and Δ^9-THCP-based inflorescences were analyzed using high performance liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) to determine their content of Δ^9-THCP and other natural and synthetic cannabinoids, thereby verifying the accuracy of the labeling.

Materials and methods

Chemicals and materials

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.

Individual standard solutions (1 mg/mL) of cannabinoids (CBGVA, CBDVA, Δ^9-THCVA, CBCVA, CBGA, CBDA, Δ^9-THCA, CBNA, CBCA, CBLA, CBGV, CBDV, Δ^9-THCV, CBCV, CBDB, Δ^9-THCB, CBG, CBD, CBC, CBL, CBCT, CBN, CBDH, and Δ^9-THCH) were purchased from Cayman Chemicals as certified reference material (CRM), while CBNVA, CBDBA, Δ^9-THCBA, Δ^9-THCA, CBDHA, Δ^9-THCHA, CBDPA, Δ^9-THCPA, *cis*-Δ^9-THC, CBGP, CBDP, Δ^9-THCP, *cis*-Δ^9-THCP, (9*R*)–HHC, (9*S*)–HHC, and CBNP were available from previous *in house* syntheses. Stock solution (0.5 mg/mL) of Δ^9-THC and Δ^8-THC were purchased from Cerilliant as CRM (Merck, Milan, Italy). All cannabinoids under investigation are

illustrated in Fig. 1. When not specified, THC homologues and isomers are intended in the *trans* configuration (6a*R*,10a*R*).

Commercial samples of gummies, cannabis flowers and vape cartridge, all declared to contain Δ^9-THCP in various amounts, were purchased from an online store based in the EU and delivered by private courier.

Sample preparation

Cannabis flower

The product is delivered in its commercial packaging (approximately 9×14 cm), consisting of a sealed closure envelope. The labels indicate "Flowers Pink Rozay; Flowers Premium THCP; 18 + only; THC < 0.2 %; THCP 90 % Quality". The hemp is declared to derive from seeds of strains listed in the Common Catalogue of Varieties of Agricultural Plant Species and the product is meant to be used for industrial, food, cosmetic, technical or horticultural purposes, but not for direct consumption or smoking. The inflorescence was in a single, compact and bright green piece, substantially devoid of seeds and twigs. Total net weight was 1.08 g (picture provided in the Supplementary Material, Figure S1).

The sample of cannabis flower was treated according to the indications of *Cannabis flos* monograph reported in the German Pharmacopoeia [25], which involved the quantitative extraction of 500 mg of the sample in 50 ml of 96 % ethanol. Specifically, 59.3 mg were extracted in 5.9 mL 96 % ethanol and the extract was diluted 1:10 with ACN and subsequently analyzed using a previously published method for the main phytocannabinoids [26] and integrated for the determination of additional cannabinoids.

Vape cartridge

The electronic cigarette cartridge (Supplementary Material, Figure S1), along with the cardboard label, was delivered in a rigid transparent plastic packaging (approximately 8×11 cm). The package displayed information regarding the product name ("Premium THCp; Cereal Milk, Sativa premium quality; 0.5 ml / THCp; Made in EU"), the ingredients ("THCp 79 %, terpene flavor 5 %. Containing no THC. High-quality THCp distillate") and the intended use ("The product is not for direct consumption but for further processing").

For the extraction of the analytes, 50 μL of the sample were diluted to 0.5 mL with 96 % ethanol. The solution was then diluted twice 1:10, the first time with 96 % ethanol and the second time with ACN. The final solution was then analyzed using the method described for the cannabis flower.

Gummy

The product was delivered in an opaque beige plastic pouch (approximately 8×13 cm) with a transparent section showing the contents (Supplementary Material, Figure S1). In addition to the product name with the ingredients ("THCP Extreme Gummies, 10 mg THCO, 2 mg THCP per gummy, 5 watermelon gummies"), the label specifies that the product is not intended for consumption.

The five gummy candies were uniform in shape, appearance, and size. They all had a slightly elongated rounded shape, a yellowish color, and the surface was variably covered with a white crystalline powder similar to sugar. Fehling assay, Molisch assay and FTIR spectroscopy were performed to identify the powder, which resulted to be sucrose. Total net weight was 20.11 g with an average weight per candy of about 4.02 g.

A portion of the candy weighing 2.065 g was cut into small pieces (2–3 mm width) and extracted with 20 mL of 96 % ethanol at room temperature for 10 min in an ultrasonic bath. The extract was then diluted 1:100 with ACN and analyzed using LC-HRMS with the previously mentioned method [26]. With the aim to evaluate the extraction efficiency, the solid residue was extracted again with ethanol in the same conditions and analyzed resulting in cannabinoid levels below the

limit of detection.

LC-HRMS method development

Ultra high-performance liquid chromatography (UHPLC) analyses were carried out for the qualitative and quantitative determinations of cannabinoids in the three sample extracts including a cannabis flower sample, gummies, and a vape cartridge. Both chromatography and mass spectrometry experimental parameters had been optimized during previous research work on cannabis derived matrices [26].

LC analyses were performed on an Ultimate 3000 ultrahigh performance liquid chromatograph (Thermo Fisher Scientific, San Jose, CA, United States), consisting of a vacuum degasser, a quaternary pump, a thermostated autosampler and column compartment. The sampler temperature was set at 4 °C and the column compartment temperature at 30 °C. A Poroshell 120 EC-C18 column (3.0 × 100 mm, 2.7 μm, Agilent, Milan, Italy) was used to separate the compounds of interest with a mobile phase composed of 0.1 % formic acid in both (A) water and (B) acetonitrile. The elution program involved an isocratic elution at 5 % ACN from 0 to 2 min, a linear gradient 5–95 % ACN in 20 min, isocratic step at 95 % ACN from 20 to 25 min, and a re-equilibration step at 5 % ACN at 5 % ACN. The injection volume was 2 μL and the flow rate was constantly kept at 0.5 mL/min. The total run time was 30 min.

The LC system was interfaced to a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, San Jose, CA, United States) equipped with a heated electrospray ionization (HESI) source. The optimized parameters were as follows: 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. Analyses were carried out using Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, United States). The exact masses of the compounds were calculated using Qual Browser in Xcalibur 3.0 software. The analyses were acquired in both FS and DDA (full scan and data-dependent acquisition) in positive and negative mode using the fast polarity switching option with a resolving power of 70,000 FWHM at m/z 200. The scan range was set at m/z 150–750 improving the sensitivity of detection; the automatic gain control (AGC) was set at 3e6, with an injection time of 100 ms. The isolation window of the quadrupole that filters the precursor ions was set at m/z 0.7. Fragmentation of precursors was performed at 30 as normalized collision energy (NCE). Identification was based on calculated $[M + H]^+$ and $[M-H]^-$ molecular ions with an accuracy of 5 ppm, retention time and fragments match (m/z and intensity). To facilitate the identification, an inclusion list was added in DDA mode with the precursor ions of the phytocannabinoid standards available (CBGVA, CBDVA, Δ^9-THCVA, CBCVA, CBGA, CBDA, Δ^9-THCA, CBNA, CBCA, CBLA, CBGV, CBDV, Δ^9-THCV, CBCV, CBDB, Δ^9-THCB, CBG, CBD, Δ^9-THC, Δ^8-THC, CBC, CBL, CBCT, CBN, CBDH, Δ^9-THCH, CBNVA, CBDBA, Δ^9-THCBA, *cis*-Δ^9-THCA, CBDHA, Δ^9-THCHA, CBDPA, Δ^9-THCPA, *cis*-Δ^9-THC, CBGP, CBDP, Δ^9-THCP, *cis*-Δ^9-THCP, (9*R*)–HHC, (9*S*)–HHC, and CBNP).

Metabolomics analyses were handled with Compound Discoverer 3.3 SP2 (Thermo Fisher Scientific). Moreover, the FISH coverage option allowed to predict the chemical structure of precursor ion and major fragments.

For quantitative purposes, linearity in the working range was evaluated as follows. A mixed stock solution of all the cannabinoids available except for Δ^9-THCP was progressively diluted in ACN to obtain six non zero calibration points at the following final concentration: 0.05, 0.10, 0.50, 1.00, 5.00, and 10.00 μg/mL. The external standard method was adopted and linear regression calculated on three replicates for each calibration point. Peak areas of each analyte (*y*) were plotted against nominal concentrations (*x*) and the linearity was assessed by the coefficient of determination (R^2) with the method of the least squares, which was greater than 0.991 for all analytes.

Method validation

Quantification of the main analyte Δ^9-THCP in the three matrices was accomplished after validation of the analytical parameters of specificity and selectivity, linear range, detection and quantification limits, (intraday and inter-day) accuracy and precision according to the "ICH Q2(R2) Guideline on validation of analytical procedures" [27].

Specificity and selectivity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present, while selectivity is the ability to differentiate the analyte of interest from other components in the sample. Therefore, the identification and/or quantitation of an analyte were demonstrated to be not impacted by the presence of other substances (e.g., impurities, degradation products, related substances, matrices, or other components likely to be present). The sample matrices were spiked with an appropriate amount of pure Δ^9-THCP in order to have a final concentration of 5 μg/mL. Untreated sample matrices were analyzed to subtract the actual concentration of Δ^9-THCP present. Then, the results were compared to the analysis of pure Δ^9-THCP standard at 5 μg/mL.

Linear range, limit of detection (LOD) and limit of quantification (LOQ)

Linearity was evaluated as a function of Δ^9-THCP concentration against peak area obtained as a response signal of the MS detector. The results were plotted by calculation of a regression line by the method of least squares, showing the coefficient of determination (R^2), y-intercept and slope of the regression line. The following five calibration points were prepared from a stock solution (100 μg/mL) and chosen to define the calibration range: 0.63, 1.25, 2.50, 5.00, and 10.00 μg/mL. Linearity was assessed with R^2 greater than 0.99 and mean precision (CV%) obtained from the back-calculated concentration within ± 15 % of the nominal concentrations the for all calibration points and within ± 20 % of the lower limit of quantification (LOQ).

The limit of detection (LOD) was estimated as a 3:1 signal-to-noise ratio (S/N). For the LOQ, a S/N of 10:1 was instead considered acceptable. In practice, LOD and LOQ were calculated using the following formulas (1) and (2) respectively:

$$LOD = 3.3*SD/s \qquad (1)$$

$$LOQ = 10*SD/s \qquad (2)$$

Where SD is the standard deviation of the response (peak areas of a blank region for five injections of a low concentrated standard) and *s* is the slope of the calibration curve. The results were validated by injecting (*n* = 3) the standard diluted at the estimated concentrations of the LOD and LOQ.

Accuracy

Accuracy measures how close results are to the true or known value and takes into account the effect of the matrix on the quantification of the analyte. For the intraday accuracy the samples were analyzed before and after spiking with three known concentrations of Δ^9-THCP (final concentrations in the matrix sample 1.0, 2.5, and 5.0 μg/mL) in three replicates. The same standard concentrations of Δ^9-THCP were also analyzed in pure acetonitrile and a new batch of calibrating dilutions was run to calculate the concentrations. The results of the standard in the pure solvent were compared to those in the spiked samples. Accuracy was calculated using formula (3):

$$A_{intraday} = 100 * C_s/C_{std} \qquad (3)$$

Where C_{std} is the nominal concentration of the spiked Δ^9-THCP standard and C_s is obtained by formula (4):

$$C_s = C_{spiked} - C_{sample} \qquad (4)$$

Where C_{spiked} is the concentration of Δ^9-THCP in the spiked sample and C_{sample} is the concentration in the unmanipulated sample.

For the inter-day accuracy, the analytical procedure was repeated in five different days and the mean concentration was compared to the concentration of the standard in pure solvent at the three levels. The inter-day accuracy was calculated according to formula (5):

$$A_{inter-day} = 100 * C_{mean}/C_{std} \qquad (5)$$

Where C_{mean} is the mean of the concentrations ($C_{s(1-5)}$) found for Δ^9-THCP in the spiked samples for five days obtained by subtracting the concentration in pure solvent according to formula (4).

The results were considered acceptable when the accuracy (both intra- and inter-day) was in the range 80–120 %.

Precision

Precision measures how close results are to one another. For the intraday precision the samples were analyzed before and after spiking with three known concentrations of Δ^9-THCP (final concentrations in the matrix sample 1.0, 2.5, and 5.0 µg/mL) in three replicates as for the accuracy experiments. The same standard solutions of Δ^9-THCP were also analyzed in pure acetonitrile and the concentrations calculated with a new batch of calibrating dilutions. Precision was reported as relative standard deviation (coefficient of variation, %CV) by calculating the standard deviation of the concentrations resulting from the spiked samples (subtracted of the concentration in the unspiked samples). Precision (%CV) was calculated using formula (6):

$$\%CV_{intraday} = 100 * SDC_s/C_{mean} \qquad (6)$$

Where SDC_s is the standard deviation of the three replicate concentrations of Δ^9-THCP calculated with formula (4) and C_{mean} is the mean concentration of the three replicates of C_s.

For the inter-day precision, the analytical procedure was repeated in five different days and the mean concentration was compared to the concentration of the standard in pure solvent at the three levels. The inter-day accuracy was calculated according to formula (7):

$$\%CV_{inter-day} = 100 * SDC_{s(1-5)}/C_{mean} \qquad (7)$$

Where SDC_{1-5} is the standard deviation of the concentrations ($C_{s(1-5)}$) found for Δ^9-THCP in the spiked samples for five days obtained by formula (4).

The results were considered acceptable when the precision (both intra- and inter-day) did not exceed ± 15 %.

Cannabinomics of retail samples

Cannabinoids and phytocannabinoids were identified employing a dedicated data analysis workflow, which was developed on Compound Discoverer software (version 3.3 SP2, Thermo Fisher Scientific) and described in a previous work [28]. Briefly, raw data from three experimental replicates and a blank sample were processed using a workflow designed as follows (Supplementary Material, Figure S2). A previously customized database of phytocannabinoids and cannabinoids complete with chemical names, 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. A customized spectral database of 42 phytocannabinoids, complete with chemical names, masses, molecular formulas, retention times and fragmentation spectra, was implemented in MZvault (ThermoFisher Scientific) for the automatic matching of m/z ratios, retention times and fragmentation spectra to compound present in the library. Moreover, parameters for predict composition were adapted to the analysis of cannabinoids and phytocannabinoids. The minimum element count 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. Exact masses extracted from the chromatograms were aligned and filtered to remove background compounds present in the blank sample, masses not present in the databases and not fragmented peaks. Lastly, MS/MS spectra of the filtered features were automatically or manually validated to assign the tentative identification according to the typical fragmentation pathways of compounds [29].

Results and discussion

Method validation

Quantification of Δ^9-THCP in retail samples was accomplished after validation of the LC-HRMS quantitative method. The separation conditions and the optimized MS parameters were taken from a previous work for the quantitative determination of eight cannabinoids in hemp material [26]. In this case, more than thirty cannabinoids were used in the method development step with the aim to achieve the best separation between all of them and provide reliable quantitative results for the investigated analyte. In particular, the linear gradient 5–95 % ACN provided the best results in terms of compound resolution and proved to be specific and selective for the intended purpose. Indeed, Δ^9-THCP resulted baseline resolved from other interfering cannabinoids or background peaks as the measurement of its concentration in cannabinoids standard mixture, extracted samples, and in a solution of the analyte alone in pure solvent was always consistent.

Quantification of Δ^9-THCP was achieved in the calibration range 0.63–10.00 µg/mL by properly diluting the extracted samples. The linearity was verified by the method of the least squares with $R^2 > 0.991$ and back calculated concentration within ± 15 % of the nominal concentration for all points and within ± 20 % for the LOQ. The results are shown in Table S2 (Supplementary Material). The LOQ was taken as the lowest point of the linear range (0.63 µg/mL) and the LOD was assessed at 0.20 µg/mL.

In the present work, calibration curves were constructed injecting solutions of the analyte in pure solvent. In a previous work, it was demonstrated that the same method suffered from no significant matrix effect and showed good recovery through a spike experiment on the Ermo variety, which does not produce phytocannabinoids [30]. In the present work, the same extensive study was not repeated and recovery and matrix effect were evaluated alongside accuracy by comparing the instrument response due to Δ^9-THCP in pure solvent (solvent-matched standards) to that obtained with spiked samples (matrix-matched standards) [31,32]. The instrument showed the same response in both cases.

Accuracy and precision were evaluated for all the three matrices for five different days. Intraday accuracy for the cannabis flower was found in the range 87.78–107.27 %, while the intraday precision varied from 1.24 % and 14.40 %; inter-day accuracy was in the range 87.90–98.47 %, while inter-day %CV ranged between 2.94 % and 13.13 %. In the gummy, intraday accuracy was found in the range 90.70–111.63 %, while the intraday precision varied from 1.67 % and 6.72 %; inter-day accuracy was in the range 86.72–109.35 %, while inter-day %CV ranged between 3.21 % and 10.43 %. In the vape cartridge, intraday accuracy was found in the range 85.85–114.57 %, while the intraday precision varied from 0.47 % and 9.28 %; inter-day accuracy was in the range 88.34–98.37 %, while inter-day %CV ranged between 2.86 % and 7.10 %. The data are reported in Table S3 (Supplementary Material) and resulted in agreement with the "ICH Q2(R2) Guideline on validation of analytical procedures" [27].

Δ^9-THCP in retail samples

The validated method was applied to the commercial samples of cannabis flower, gummy and vape cartridge, which were declared to contain a certain amount of Δ^9-THCP: "90 % quality" in the cannabis flower, 2 mg in each gummy, and 96 % in the vape cartridge. The analyses of ten replicates showed an amount of Δ^9-THCP (Table 1) of

Table 1
Amount of Δ^9-THCP found in the retail samples compared to the expected amount declared on the label. Values are reported as mean ± sd (n = 10).

Matrix	Amount found	Expected amount
Cannabis flower	0.497 ± 0.032 % (*w/w*)	90 % (*w/w*)
Gummies	6.27 ± 0.74 µg/g	500 µg/g
Vape cartridge	0.504 ± 0.017 mg/mL	790 mg/mL

0.497 % (*w/w*) in the cannabis flower, 6.27 µg/g in the gummies (12.54 µg in each gummy), and 504 µg/mL in the vape cartridge (252 µg in the entire cartridge of 0.5 mL, about 0.05 %). The results were not in compliance with what reported in the label, but rather far below the amounts declared: almost 200-fold lower in the flower, about 80-fold lower in the gummies and over 1500-fold below the declared amount in the vape cartridge.

Cannabinomics of retail samples

All samples were subjected to LC-HRMS analysis using an untargeted metabolomics approach, which enabled the tentative identification of numerous cannabinoids of both natural and synthetic origin. Over fifty compounds including isomeric forms were identified with the richest sample being the vape cartridge, followed by the flower and the gummy, the latter being the one showing the poorest pool of cannabinoids. Δ^9-THCP was quantified using the *ad hoc* validated method, while the other identified cannabinoids were quantified using the calibration curves built using the respective analytical standards.

Cannabis flower

The LC-HRMS chromatogram of the cannabis flower showed the typical profile of a plant extract with predominant amount of the carboxylated species, especially CBDA (7.924 %, based on dry wight), THCA (0.497 %) and its isomer *cis*-THCA (0.027 %), CBGA (0.468 %), CBCA (0.318 %), and CBNA (0.109 %), followed by the minor phytocannabinoids represented by CBDA and CBCA homologs with different alkyl chain length, including in order of relative abundance CBDBA (0.068 %), CBDVA (0.025 %), CBDHA (0.003 %), CBCVA (0.003 %), and CBDPA (0.002 %). The methyl homologue of CBDA was also detected and putatively identified in the chemical profile of the cannabis flower by comparison of its HRMS spectrum with those of the other CBDA homologues. Amongst the decarboxylated cannabinoids Δ^9-THCP (0.497 %) was found as the most abundant, followed by its oxidation product CBNP (0.459 %), and CBD (0.345 %). Other minor species were represented by the *cis* isomer of Δ^9-THCP (*cis*-Δ^9-THCP 0.038 %), Δ^9-

THC (0.022 %) and its *cis* isoform (0.030 %), CBG (0.053 %), CBL (0.020 %), and CBDP (0.018 %).

Considering the ratio of the carboxylated/decarboxylated form for all natural cannabinoids, the cannabis flower had only been partially decarboxylated or eventually dried to remove the moisture content. On the other hand, the sole presence of the decarboxylated form of THCP suggested a synthetic origin of such compound and its later addition to the plant product.

In addition to the plethora of the aforementioned phytocannabinoids, other cannabinoids of synthetic or semi-synthetic source were identified besides Δ^9-THCP. In particular, THC acetate, H2-CBD, and the two epimers of HHC, of which the *R* form was the predominant one (2.4:1 *er*, *R/S*). Indeed, the concentrations of the two epimers resulted 0.020 % and 0.009 % for the *R* and *S* form, respectively. The fragmentation pattern of acetylated derivatives of cannabinoids (Fig. 2) are easily recognizable by the loss of the $CH_3C = O$ group generating two adjacent fragments corresponding to the loss of 43.05 and 42.05. In particular, the $[M + H]^+$ precursor ion of THC acetate (22.25 min) is 357.2475 and the main fragments were found at *m/z* 315.1955 and 314.1875.

In general, the acetylated versions of standard cannabinoids like CBD, THC, THCP, and so forth, are characterized by a slight increase in the lipophilicity with respect to their parent compounds with consequent advantage in terms of both entering the central nervous system and being protected from metabolic inactivation by conjugation or oxidation [33].

H2-CBD, a molecule generated by the partial hydrogenation of CBD, was found in the chromatogram at 19.74 min with the same $[M + H]^+$ as CBG and HHC, but fragmentation pattern more similar to that of HHC (Fig. 3). HHC and H2-CBD can be distinguished by two main features in their HRMS spectra. The first difference consisted of the relative abundance of the precursor ion at *m/z* 317.2475, which represented the base peak in the HHC spectrum, while it had low abundance in the H2-CBD spectrum. Second, the fragment at *m/z* 137.1326 in the HHC spectrum corresponded to the terpene moiety and had a low abundance; on the other hand, the fragment at *m/z* 137.0599 in the H2-CBD spectrum corresponded to the resorcinol moiety with two additional carbon units and represented the base peak.

Interestingly, a peak eluting at 18.21 min with $[M + H]^+$ at *m/z* 375.2524 and showing a fragmentation pattern with two main fragments at *m/z* 251.1278 and 209.1172 and very low precursor ion was tentatively identified as the methyl ester of a partially hydrogenated CBDA (H2-CBDA methyl ester), a CBDA homolog of synthetic source (Fig. 4). The low-abundance fragments at *m/z* 357.2049 and 339.1955 suggested the presence of two free hydroxyl groups, while the fragment
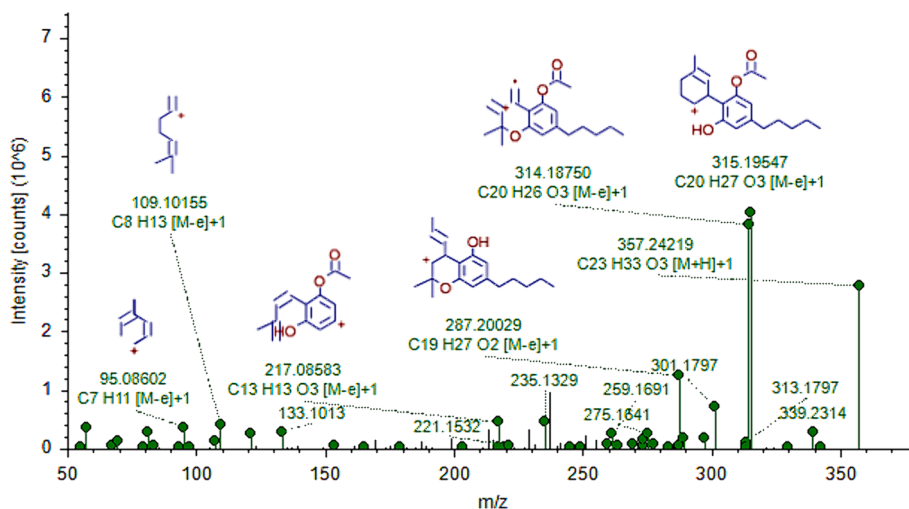

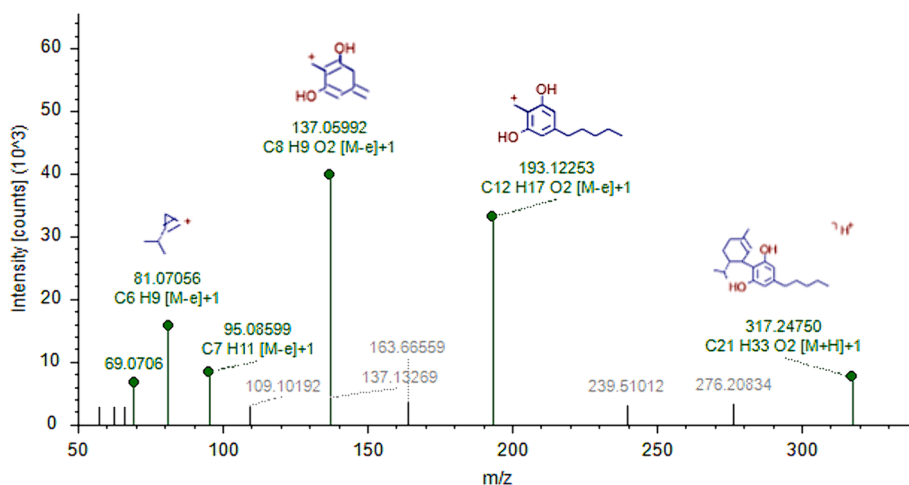**Fig. 2.** High-resolution mass fragmentation spectrum in HESI + mode of THC acetate.

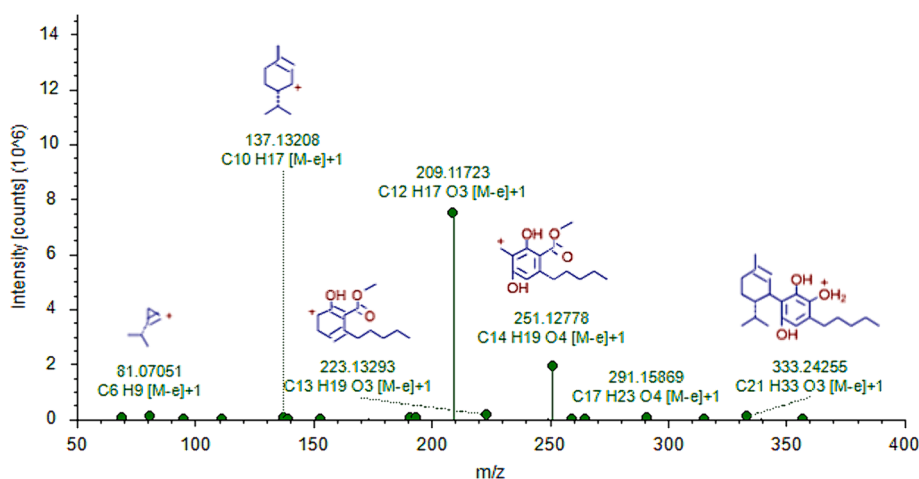**Fig. 3.** High-resolution mass fragmentation spectrum in HESI + mode of H2-CBD.

Fig. 4. High-resolution mass fragmentation spectrum in HESI + mode of H2-CBDA methyl ester.

at m/z 333.2425 seemed to derive from the loss of a $CH_2C = O$ from the methyl ester group. The fragment at m/z 251.1278 could be generated by the loss of the terpene moiety, while the fragment at m/z 209.1172 added the further loss of the methoxy group of the methyl ester. According to the fragmentation pattern, CBDA could have been partially hydrogenated at the C8-C9 position of the isobutylene moiety and not at the C1-C2 position as the latter case would have generated two epimers.

Many other unknown peaks could be observed in the LC-HRMS chromatogram, although their identification was not attempted due to the difficulty in interpreting their MS spectrum.

Gummy

The chromatogram of the gummy sample showed the prevalence of HHC, particularly of the *R* epimer (1.8:1 *er*, *R/S*), and CBDP, while only traces of Δ^9-THCP were found. HHC was surprisingly found at the remarkable concentrations of 208 μg/g and 118 μg/g for the *R* and *S* epimer respectively, while CBDP was found at 53 μg/g and Δ^9-THCP at 6.27 μg/g, far below the amount specified in the label (2 mg per gummy or 500 μg/g).

Vape cartridge

The vape cartridge showed an interesting and rich cannabinoid profile in the LC-HRMS chromatogram, mainly represented by unknown compounds. The major peak in the total chromatogram corresponded to H4-CBD, followed by Δ^9-THCP, and HHC with the *R* form being the

dominant epimer (1.6:1 *er*, *R/S*). Additionally, another major peak was represented by a compound eluting at 19.11 min with $[M + H]^+$ at m/z 321.2784. Δ^9-THCP reached a concentration of 504 μg/mL, while the two epimers of HHC were found at 5.42 mg/mL and 3.46 mg/mL. A smaller amount of CBDP (0.21 mg/mL) was also present in the vape cartridge, probably derived from the synthetic process used to obtain Δ^9-THCP. Indeed, the $[M + H]^+$ of Δ^9-THCP showed a complex profile as it probably underwent a poor purification showing the characteristic impurity pattern consisting of traces of CBDP, *cis*-Δ^9-THCP, $\Delta^{9,11}$-THCP, and Δ^8-THCP, the latter being the most abundant impurity.

H4-CBD showed a low $[M + H]^+$ precursor ion at m/z 319.2630 and eluted at 18.78 min in the LC-MS chromatogram with a small shoulder at 18.69 min, which likely corresponded to its stereoisomer (epimer at C1 of the terpene moiety). Very few fragments characterized its spectrum (Fig. 5), among which the one at m/z 181.1223 with low abundance corresponded to olivetol and the base peak at m/z 83.0861 corresponded to a broken terpene moiety with formula $[C_6H_{11}]^+$.

The peak eluting at 19.11 min with $[M + H]^+$ at m/z 321.2784 was identified as H6-CBD, a molecule similar to H4-CBD with a cyclohexanone in place of the resorcinol ring. The HRMS spectrum showed two fragments at m/z 303.2680 and 285.2576 corresponding to the loss of two hydroxyl groups from the tautomeric dienol structure (Fig. 6). The loss of one hydroxyl group and the breakage of the alkyl side chain at the C1″-C2″ position generated the fragment at m/z 247.2055. The remainder fragments were very low and corresponded mainly to
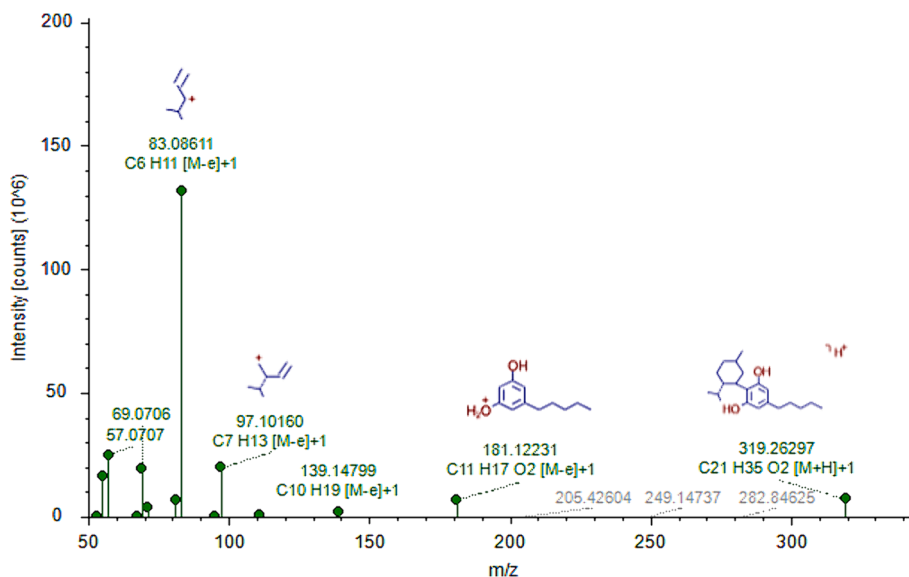
Fig. 5. High-resolution mass fragmentation spectrum in HESI + mode of H4-CBD.
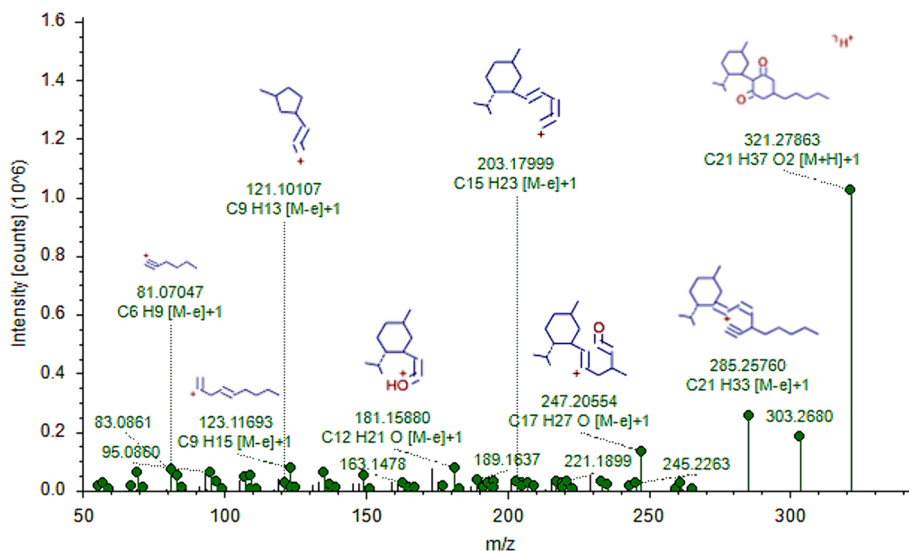

Fig. 6. High-resolution mass fragmentation spectrum in HESI + mode of H6-CBD.

fragments of the terpene moiety. As for H4-CBD and HHC, this molecule appeared as two peaks, the major one at 19.11 min and a minor peak at 18.98 min, which likely corresponded to the epimer at C1 of the terpene moiety. The formation of such a species could be attributed to further reduction of the resorcinol aromatic ring during the catalytic hydrogenation of cannabinoids. This reaction is known to occur with the use of hydrogen gas under pressure assisted by the presence of a catalyst, generally Pd/C, and alkaline conditions to achieve high selectivity, although non-alkaline conditions can also lead to a complete hydrogenation of the resorcinol ring [34].

The $[M + H]^+$ precursor ion at *m/z* 317.2475 is characteristic not only of CBG and HHC but also of H2-CBD, which was found as three main peaks eluting at 19.34 min, 19.90 min, and 20.33 min. The peak of H2-CBD identified in the cannabis inflorescence at 19.74 min represented only a small peak in the vape cartridge. All peaks showed similar fragmentation pattern differing only in the relative abundance of the fragments.

Other cannabinoids putatively identified included several CBD and THC acetate isomers, whose structure elucidation was of difficult interpretation. Many other unknown compounds were present in the chromatogram, but their identification could not be accomplished due to the lack of reference spectral data and poor fragmentation. The information from the exact mass, *m/z* of the fragments and retention time suggested their structure could be likely attributable to partially or totally hydrogenated cannabinoids and acetylated derivatives, such as H2-THC acetate (22.81 min, *m/z* 359.2575), H2-CBD diacetate (20.03 min, *m/z* 401.2683), H4-HU-331 (minor peak at 21.85 min and major peak at 21.98 min, *m/z* 331.2279), HHCP (22.59 min, *m/z* 345.2782), and others.

Conclusions

Out of the three samples examined, two contained Δ^9-THCP in significant amount, but not as the main compound and not in quantities corresponding to what is declared on the label. Specifically, the vape cartridge, in addition to the previously mentioned quantities of Δ^9- and Δ^8-THCP, mainly contained H4-CBD. The gummy candy contained only traces of THCP and mainly the CBDP isomer, along with appreciable

amounts of *R*- and *S*-HHC. The cannabis flower contained all the carboxylated phytocannabinoids typical of industrial hemp, among which the non-compliant level of Δ^9-THC stood out. The presence of reaction by-products indicates a synthetic origin of the added cannabinoids, particularly the presence of CBDP in the candy, which can be justified by the THCP synthesis process, and of hydrogenated and acetylated products in the vape cartridge. The latter represent a new trend of increasingly growing success in the cannabis market

In all cases, the label does not accurately reflect the exact composition found through analysis. This highlights the inherent danger of consuming such products since consumers, unaware of the compounds they are ingesting and their actual doses, could experience unexpected reactions. Lastly, the presence of compounds subject to legal restrictions such as HHC or THCA exceeding legal limits would put the possessor/consumer in a position of having violated a criminal law in case of verification by regulatory authorities.

CRediT authorship contribution statement

Cristian Caprari: Validation, Investigation, Formal analysis. **Elena Ferri:** Validation, Investigation, Formal analysis. **Martin G. Schmid:** Conceptualization. **Loretta L. Del Mercato:** Funding acquisition. **Cinzia Citti:** Writing – original draft, Supervision, Project administration, Methodology, Investigation, Data curation. **Giuseppe Cannazza:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Cinzia Citti, Giuseppe Cannazza has patent pending to Consiglio Nazionale Delle Ricerche 36 If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.forc.2024.100595.

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