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Article

Analysis of Isomeric Cannabinoid Standards and *Cannabis* Products by UPLC-ESI-TWIM-MS: a Comparison with GC-MS and GC × GC-QMS

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The *Cannabis sativa* L. plant has a complex chemical composition, containing various chemical compounds such as terpenes, sugars, hydrocarbons, steroids, flavonoids, and amino acids. Few works have attempted to identify the constitutional isomers of cannabinoids that are found in the plant. The present study reported the analysis of seven cannabinoid standards: five neutral and two acidic, as well as *Cannabis* products (hashish and marijuana) and parts of the *Cannabis* plant (flower and leaf) using mono-dimensional gas chromatography coupled with mass spectrometry (GC-MS) and two-dimensional gas chromatography coupled with quadrupole MS (GC × GC-QMS), ultra performance liquid chromatography coupled with electrospray ionization-quadrupole-time of flight (UPLC-ESI-QTOF)-MS and UPLC-ESI-travelling wave ion mobility (TWIM)-MS. The results of GC-MS demonstrated close retention times (Δt_R = 1.303 min) in separation of the five cannabinoid standards, whereas GC × GC-QMS provided a substantially better identification and distinction of constitutional isomers of cannabinoids, where a total of 11 cannabinoids were identified in the hashish sample. UPLC-QTOF-MS and UPLC-TWIM-MS data obtained complete chemical information, in which ESI(+) revealed the presence of seven constitutional isomers of Δ^9-tetrahydrocannabinol (Δ^9-THC), whereas ESI(–) proved the presence of four isomers of Δ^9-tetrahydrocannabinolic acid A (Δ^9-THCA A).

Keywords: cannabinoids, ion mobility, isomers, Δ^9-THC, mass spectrometry

Introduction

The *Cannabis sativa* L. plant is cultivated and consumed in most regions of the world.[1] It is an annual, dioecious herb belonging to the family of Cannabacea, *Cannabis* genus,[2] that has a history of pharmacological studies and is used for recreational purposes.[3,4]

Also known as marijuana, *Cannabis* has a complex chemical composition that includes terpenes, sugars, hydrocarbons, steroids, flavonoids, amino acids, among others.[5] Presently, more than 700 natural constituents of the plant have been identified,[6] of which more than 100 are classified as cannabinoids,[7,8] which are concentrated on resinous secretions produced by glandular trichomes and primarily distributed on the aerial surfaces of the plant and the female inflorescences.[7]

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The most abundant cannabinoids present in the *Cannabis* plant are:[9,10] (*i*) cannabidiol (CBD), an anticonvulsant drug tested in treatments of epileptic patients,[11] in addition to being anxiolytic, anti-inflammatory, antipsychotic, antispasmodic and analgesic;[12] (*ii*) cannabigerol (CBG), which has antiproliferative and antiglaucoma activities[13] as well as antibiotic, anti-inflammatory, antifungal and analgesic activities;[12] (*iii*) cannabinol (CBN),[1] a sedative and anticonvulsant that is anti-inflammatory;[12] (*iv*) cannabichromene (CBC), which is anti-inflammatory, antifungal and analgesic;[12] and (*v*) Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive compound of the *Cannabis* plant.[1,14-18]

Most cannabinoids have a 21 carbon atom structural feature, with possible variations in the length of their side chains (C1-C5) attached to the aromatic ring. In the most common homologs, the *n*-pentyl side chain is replaced by *n*-propyl, and these analogues are named using the suffix "varin", for example, Δ^9-tetrahydrocannabivarin (Δ^9-THCV), cannabidivarin (CBDV) and cannabinovarin (CBNV).[16]

Our group has explored the identification of constitutional isomers of cannabinoids that are found in *Cannabis*.[17,18] Figures 1a-1c illustrate some of these compounds as cannabinodiol (CBND), CBN and cannabifuran (CBF) with molecular formula (M) = $C_{21}H_{26}O_2$, double bond equivalent (DBE) of 9 and an average molecular weight (M_w) of 310 Da (Figure 1a); CBD, CBC, cannabicyclol (CBL), Δ^9-*trans*-THC, Δ^9-*cis*-THC and Δ^8-*trans*-tetrahydrocannabinol (Δ^8-THC), with M = $C_{21}H_{30}O_2$, DBE 7, and an M_w of 314 Da (Figure 1b); and the cannabidiolic (CBDA), cannabinchromenic (CBCA), cannabicyclolic (CBLA), Δ^9-tetrahydrocannabinolic A and B (Δ^9-THCA A and B, respectively) and Δ^8-tetrahydrocannabinolic (Δ^8-THCA) acids, M = $C_{22}H_{30}O_4$, DBE 8, and M_w = 358 Da (Figure 1c).

In fresh *Cannabis* plant material, most cannabinoids produced by plant metabolism are in the form of carboxylic acids, such as Δ^9-THCA A, CBDA, and cannabigerolic acid (CBGA). They can be converted into their analogues by decarboxylation, via loss of a COOH group, producing "neutral cannabinoids" such as Δ^9-THC, CBD, and CBG.[3,9,10]

For forensic analysis, the study of the chemical composition of *Cannabis* and its products is considered relevant to the identification of the chemical profiles to trace possible traffic routes.[19-22] Several factors in addition to the genetic characteristics can influence the chemical composition of the cannabinoids in the plant, namely, the climate, light, humidity, elevation of the cultivated region, etc.[23]

The development of new analytical methodologies for cannabinoid analysis has been an advance in forensic toxicology.[21] An example of this is gas chromatography coupled to mass spectrometry (GC-MS), which allows the separation and identification of cannabinoids from their National Institute of Standards and Technology (NIST) library.[1] However, it is not possible to detect terpenophenolic acids, which possess labile groups and are unstable in the GC column, undergoing thermal conversion reactions and producing neutral cannabinoid species or their degradation products.[8,24]

Another technique analogous to GC-MS, with a greater chromatographic resolution power, is comprehensive two-dimensional (2D) gas chromatography (GC × GC) technique.[25,26] The GC × GC coupled with quadrupole MS

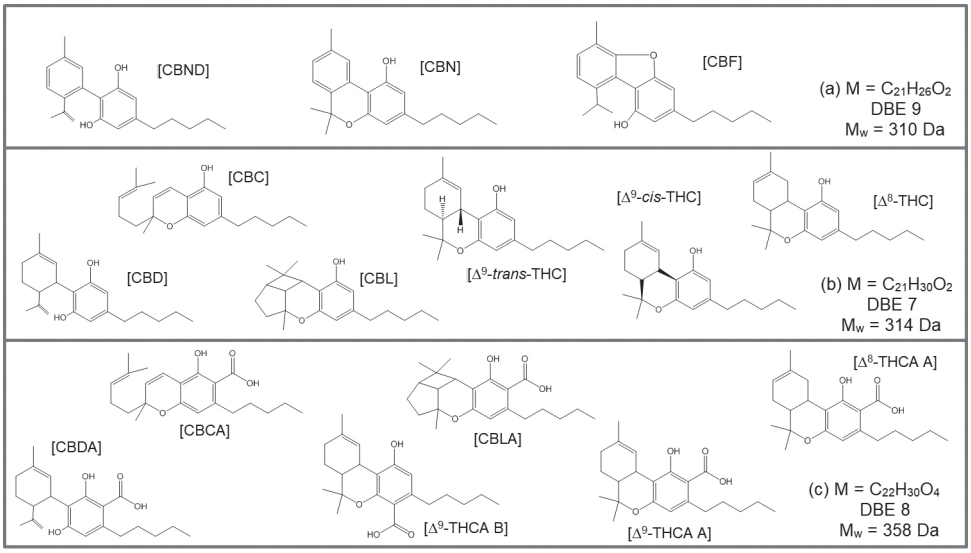

Figure 1. Cannabinoid isomers of (a) CBN, (b) Δ^9-THC and (c) Δ^9-THCA and their respective molecular formulas (M), DBE and M_w values.

(QMS) technique is applied to the identification of complex matrices, being able to identify biomarkers and cannabinoid isomers, primarily those described in Figures 1a and 1b.[25-27] An alternative would be derivatization reactions, but the addition of this step makes the forensic routine even more laborious.[25,27]

Unlike GC × GC-QMS and GC-MS techniques, *Cannabis* analysis using liquid chromatography coupled with mass spectrometry (LC-MS) allows the complete identification of all cannabinoids in the neutral and acid forms, making possible unambiguous chemical profiles, without the need for derivatization reaction.[8,28-30] The LC-MS technique has already been used to identify and quantify cannabinoids in human fluids[31,32] and *in natura* samples[33] or with minimal processing samples, such as marijuana.[34,35]

As a complement to the LC-MS technique, the development of ion mobility spectrometry (IMS) allows the differentiation of ions by their sizes and/or spatial conformations, separating in the gas phase according to the diffusion time across a mobility cell. IMS is an extremely versatile technique, providing a new dimension of data and the possibility of using different ionization sources when combined with LC-MS systems.[36]

Recent studies report the use of the travelling wave ion mobility mass spectrometry (TWIM-MS) technique for the distinction of isomers in complex samples. Romão *et al*.[37] used positive mode electrospray ionization (ESI(+)) TWIM-MS to distinguish three isomers of chlorophenylpiperazine (*o*-CPP, *m*-CPP, and *p*-CPP) using CO_2 as a drift gas. Gwak and Almirall[38] developed a study using 35 new psychoactive substances, characterized by IMS based on a drift time (Dt) IMS. Benigni *et al*.[39] used the selected accumulation trapped ion mobility spectrometry (SA-TIMS) method coupled with Fourier transform ion cyclotron resonance (FT-ICR) MS for the separation and characterization of hormones in complex environmental matrices. Isobars were primarily identified in a complex matrix of water-soluble organic matter, and among them were α-estradiol, bisphenol A, and 17-ethynylestradiol.

Recently, Tose *et al*.[40] used ultra performance liquid chromatography (UPLC) coupled to TWIM-MS as an analytical tool capable of identifying isomeric compounds in *Cannabis* products such as hashish, marijuana, and parts of the *Cannabis* plant (flower and leaf). The results showed the separation of several isomeric compounds in the single ion acquisition mode (SIM), however, the unambiguous identification of all isomers was compromised due to the similarity between fragmentation profiles and the deficiency of reference material.

In this context, the present work describes the application of the GC-MS, GC × GC-QMS and UPLC-ESI-TWIM-MS techniques in the characterization of five neutral cannabinoid standards (Δ^9-THC, CBD, CBG, CBC and CBN), and two standards of terpenophenolic acid precursors (Δ^9-THCA A and CBDA). The data obtained were compared to *Cannabis* products such as hashish and marijuana samples, and parts of the plant (leaf and flower).

Experimental

Samples and reagents

Seven certified cannabinoid standards (Δ^9-THC, CBD, CBC, CBN, CBG, Δ^9-THCA A and CBDA), supplied by Cerilliant at concentrations of 1 mg mL^{-1}, were dissolved in methanol (neutral cannabinoids) or acetonitrile (acid cannabinoids). Methanol and acetonitrile (analytical purity grade higher than 99.5%) were purchased from Vetec. The solutions were maintained at 8 °C until analysis.

Cannabis products (marijuana and hashish) and parts of the plant (flower and leaf) were supplied by the Civil Police of Espírito Santo (CP-ES), Vitória, Brazil, through a cooperation agreement, process No. 23068.011398/2012-72. The samples were weighed (ca. 10 mg) and solubilized in methanol.

GC-MS

Five neutral cannabinoid standards solutions (Δ^9-THC, CBD, CBC, CBN, and CBG) were individually analyzed by GC-MS (Agilent Technologies, 7890B). The column used was DB5 (30 m × i.d. 0.25 mm × 0.25 µm, 5% diphenyl-95% dimethylpolysiloxane; J & W Scientific, Agilent Technologies). Helium gas was used as an eluent, with a constant flow of 1 mL min^{-1}. The injector was maintained in a 1:10 split ratio mode at 280 °C. The initial oven temperature varied from 80 °C (2 min) to 290 °C (5 min), with a heating rate of 10 °C min^{-1}, and operation was in full scan mode in the range of *m/z* 50-400.

For the analysis of the standards, 7 µL of each solution were collected, and the solvent was evaporated. The samples were then resuspended in 80 µL of dichloromethane (Vetec, purity of 99.5%).

GC × GC-QMS

Seized samples of hashish and parts of *Cannabis* plant (flower and leaf) were analyzed using a GC × GC-QMS (GCMS QP 2010 Shimadzu ULTRA system) containing an autosampler AOC-5000 Plus equipped with a ZX1 modulator (Zoex). The modulator uses a simple nitrogen jet system. Chromatographic separation in the first dimension

was performed on non-polar column, DB5 (5% phenyl, 95% polymethylsiloxane, 30 m × i.d. 0.25 mm × 0.25 μm; J & W Scientific, Agilent Technologies). In the second dimension a medium-polar column, DB-17 (50% phenyl and 50% methylpolysiloxane, 1.8 m × i.d. 0.1 mm × 0.1 μm; J &W Scientific, Agilent Technologies) was used. The oven temperature program operated from 80 °C (5 min) to 300 °C (10 min) with a heating rate of 7 °C min^{-1}. The injector temperature was maintained at 280 °C in the splitless mode, and 1 μL of each solution was injected. Helium was used as the carrier gas, with a flow rate of 1 mL min^{-1}, and the interface temperature and ion source were maintained at 300 °C. The acquisition range was *m/z* 50 to 550 and the processing was performed by GC Image software (Zoex), using the NIST05 library to identify the compounds.

UPLC-ESI-TWIM-MS and ESI-tandem mass spectrometry (MS/MS)

Three isomers of M_w = 314 Da (Δ^9-THC, CBD, and CBC) and two isomers of M_w = 358 Da (Δ^9-THCA A and CBDA) were initially analyzed by a chromatographic system composed of a Waters Acquity UPLC I-Class coupled to a Waters Synapt G2-S TWIM-MS high definition mass spectrometer (HDMS). Posteriorly, hashish samples, parts of the *Cannabis* plant and marijuana were analyzed using the UPLC-MS system and UPLC-TWIM-MS in the full scan and extracted ion chromatograms (XIC) mode acquisition.

The Synapt G2-S HDMS has a hybrid mass analyzer, which includes a quadrupole (Q) and time of flight (TOF) analyzer. Chromatographic elution used a binary solvent with mobile phase (phase A = 0.1% v/v water/formic acid, phase B = 0.1% v/v methanol/formic acid). The flow rate was 0.50 μL min^{-1}. The conditions of analysis were as follows: 10% phase B in 0 min; 60% phase B in 8 min; 95% phase B in 10 min; 95% phase B in 12 min; and after 2 min, the analysis returned to the initial condition. The TOF analyzer was operated with a resolution power of ($m / \Delta m_{50\%}$) = 45,000 and calibrated with 0.1% sodium trifluoroacetate (NaTFA) in 1:1 (v/v) acetonitrile/water over 100-700 *m/z*.

The ion-transfer and ion-accumulation cells were operated at a pressure of 10^{-2} mbar of argon. N_2 gas was used in the mobility and ion separation experiments. Data acquisition and processing were performed in MassLynx 4.1 software (Waters). The parameters of the ESI source and ion mobility cell were: (*i*) capillary voltage: 2.5-3.5 kV; (*ii*) source temperature: 90 °C; (*iii*) cone voltage: 40-50 V; (*iv*) desolvation gas flow (N_2): 500 L h^{-1}; (*v*) accumulation rate: 0.1 s $scan^{-1}$; (*vi*) travelling wave height: 29.0-40.0 V; (*vii*) travelling wave speed: 650-652 m s^{-1}; and (*viii*) nitrogen pressure (drift gas): 2.90 mbar.

ESI(+) and ESI(−)MS/MS experiments were performed using collision energies of 15-30% for the ions of *m/z* 315 (CBD, CBC, and Δ^9-THC standards; Figure 1b) and *m/z* 357 (Δ^9-THCA and CBDA standards; Figure 1c).

Results and Discussion

GC-MS

The chromatograms of the Δ^9-THC, CBD, CBC, CBN and CBG standards are shown in Supplementary Information (SI) section Figures S1a-S1e, respectively, and their respective electron ionization (EI)-MS spectra in Figure S2 (SI section), in which extremely close retention times (Δt_R = 1.303 min) are observed. In all cases, the acquired peaks are well defined, and the order of elution is similar to that reported in the literature, CBD < CBC < Δ^9-THC < CBG and CBN.[1,10,41]

The similarity of each cannabinoid with its own NIST library standard spectrum ranged from 45 to 98%. With the exception of CBG (M_w = 316 Da) and CBN (M_w = 310 Da), the cannabinoids are constitutional isomers (M_w = 314 Da).

When analyzed for the fragmentation profiles between the isomers of M_w = 314 Da, the EI-MS spectrum of Δ^9-THC (Figure S1) is unequivocal, differing from the CBD and CBC molecules, even the former presenting similar chemical connectivity (*m/z* 259, 193 and 123). Conversely, the CBD and CBC isomers, which have a distinct connectivity in their structures, present a similar fragmentation spectrum (*m/z* 299, 271, 258, 243 and 231), as shown in Figures S2b and S2c (SI section), and contain the base peak of *m/z* 231. In addition, they have a very close elution time (Δt_R = 0.017 min). Mariotti *et al.*,[1] using GC-MS, reported a similar retention time between the cannabinoids CBD and CBC.

The similar retention time observed among cannabinoids in the GC-MS analysis is related to the type of stationary phase that is used (DB5 column, 5% diphenyl-95% dimethylpolysiloxane). This stationary phase is classified as a non-polar column, thus providing a lower interaction with the analytes. If a column of higher polarity or a PMPS-5 column were used, a better separation should be expected primarily between the constitutional isomers, CBD and CBC, the latter isomer having the shorter retention time.

A longer elution time was observed for CBN (DBE 9, being composed of a tri-cyclic and bi-aromatic system), as can be seen in Figure S1e (SI section). Aromatic compounds produce π-stacking intermolecular interactions (stacking of the chain rings) between the stationary phase and the analyte, which increase the retention time.

GC × GC-QMS

GC × GC-QMS provides a better chromatographic resolution in relation to the one-dimensional separation system.[42] Therefore, GC × GC-QMS was used in the analysis of three samples seized by the police (hashish, flower, and leaf), and their respective chromatograms are shown in Figures 2a-2c.

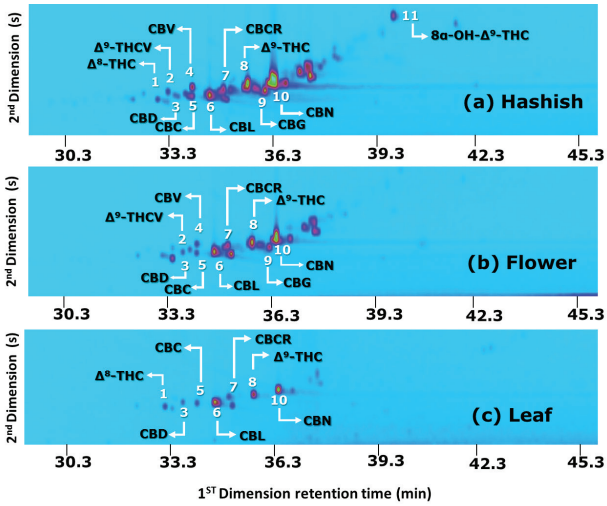

Figure 2. Chromatograms obtained from GC × GC-QMS analysis of (a) hashish, (b) flower and (c) leaf.

A total of 11 cannabinoids (Δ^8-THC, Δ^9-THCV, CBD, cannabivarin (CBV), CBC, CBL, cannabicumarone (CBCR), Δ^9-THC, CBG, CBN, and 8α-OH-Δ^9-THC) were identified in the hashish (Figure 2a), whereas a smaller number of compounds was observed in flower and leaf samples (9 and 7, respectively, Figures 2b and 2c). The Δ^9-THC and CBN cannabinoids are the most abundant species. The scientific names of the cannabinoids and their respective M_w and similarity values (obtained from the NIST05 library) are shown in Table 1.

Among the cannabinoids detected, the molecules of Δ^8-THC, CBD, CBC, and CBL are highlighted as constitutional isomers of Δ^9-THC (M_w = 314 Da), and they elute in the following order: Δ^8-THC < CBD < CBC < CBL < Δ^9-THC.

The *Cannabis* flower is considered the part of the plant that contains a high content of Δ^9-THC.[2,5] Similarly, hashish samples also have a high content of Δ^9-THC because it is produced from the resinous secretions of the plant that are associated with floral structures.[2] In addition to the cannabinoids, these samples contain a wide variety of terpenes, sugars, and flavonoids, typically present in *Cannabis* plants.[1]

In fresh plant material, most cannabinoids are available in the form of their acid precursors, which are subsequently converted by decarboxylation processes to their corresponding neutral cannabinoids. Decarboxylation can occur over time under heating or alkaline conditions.[9] The cannabinoid Δ^9-THC has the Δ^9-THCA A as its main precursor molecule, and its decarboxylation occurs from 125 to 150 °C.[1,4,43,44] Considering the direct injection conditions of GC × GC-QMS (temperature = 280 °C), and the absence of derivatization methods of terpenophenolic acids,[45-47] in this work, the GC × GC-QMS method was not suitable for the identification of acid species present in Figure 1c.

The cannabinoid CBN is produced by degradation of Δ^9-THC and does not occur naturally in the plant.[5,11,12] The greater abundance of CBN could be justified due to the ageing or storage conditions of the raw sample that was

Table 1. Cannabinoids identified in hashish, flower and leaf samples from the GC × GC-QMS data as well as their respective M_w and similarity values

Name		Ion No.	M_w / Da	NIST similarity / %	Hashish	Flower	Leaf
Δ^8-THC	Δ^8-tetrahydrocannabinol	1	314	82	×	–	×
Δ^9-THCV	Δ^9-tetrahydrocannabivarin	2	286	78	×	×	–
CBD	cannabidiol	3	314	75	×	×	×
CBV	cannabivarin	4	282	78	×	×	–
CBC	cannabichromene	5	314	86	×	×	×
CBL	cannabicyclol	6	314	80	×	×	×
CBCR	cannabicumarone	7	328	66	×	×	×
Δ^9-THC	Δ^9-tetrahydrocannabinol	8	314	89	×	×	×
CBG	cannabigerol	9	316	83	×	×	–
CBN	cannabinol	10	310	84	×	×	×
8α-OH-Δ^9-THC	8α-hydroxy-Δ^9-THC	11	326	70	×	–	–

M_w: average molecular weight; NIST: National Institute of Standards and Technology; ×: detected; –: non-detected.

seized, and to conditions of injection. Moreover, other phenomena (such as extended periods of storage and light exposure) may potentiate the degradation of Δ^9-THC to generate CBN.[48]

UPLC-ESI(+)-QTOF-MS, and ESI(+)-MS/MS

The chromatographic profile of Δ^9-THC and its isomers (CBD and CBC) were acquired in the full scan mode by UPLC-ESI(+)-QTOF-MS (Figures 3a-3c). Note that the three isomers of *m/z* 315.2354 were detected in the protonated form, $[M + H]^+$ ion, with retention times of 5.71 min (CBC), 5.21 min (Δ^9-THC) and 3.86 min (CBD).

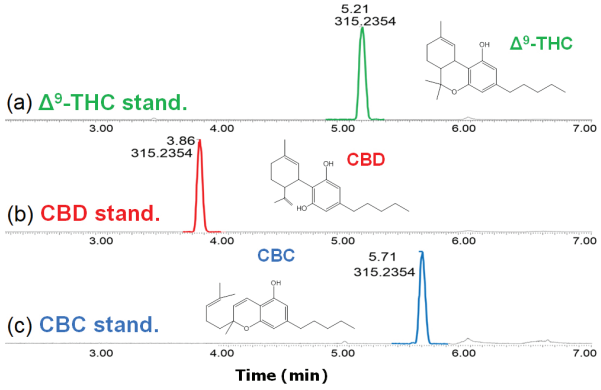

Figure 3. Chromatograms obtained from UPLC-ESI(+)-QTOF-MS analysis of cannabinoid standards of *m/z* 315: (a) Δ^9-THC, (b) CBD and (c) CBC.

Collision-induced dissociation (CID) experiments were performed to confirm the structure and connectivity of each isomer, and the ESI(+)MS/MS spectra for the CBD, Δ^9-THC and CBC molecules are shown in Figures 4a-4c, respectively, along with their respective fragmentation mechanisms (*m/z* transitions of 315 → 259, 315 → 193 and 193 → 123). Constitutional isomers present similar fragmentation profiles,[40,49-51] in which the signals of *m/z* 259, 193 and 123, are highlighted as main fragments, being similar to those reported for a typical marijuana sample seized by forensic police.[17,40]

The neutral loss of 56 Da (*m/z* 315 → 259 transition) can be represented by cleavage of the side chain of the cannabinoid molecule (pentyl group, C_4H_8) resulting in the $[C_{17}H_{22}O_2 + H]^+$ fragment (structure (I), Figure 4). Another fragmentation pathway can be justified by the transition of *m/z* 315 → 193, with a neutral loss of 122 Da (C_9H_{14}). After breaking the C–O bond (ether function) in Δ^9-THC and CBC molecules, fragment (II) may be formed during the protonation stage and cleavage of the terpene ring (Δ^9-THC and CBD). Subsequently, there is a neutral loss of 70 Da (*m/z* 193 → 123 transition) corresponding to the elimination of the side chain of the molecule (C_5H_{10}, pentene) and detection of fragment (III), $[C_7H_6O_2 + H]^+$ ion. A reaction mechanism for the fragments of the isomeric standards (CBD, Δ^9-THC, and CBC) is proposed in more detail in Figures S3a-S3d (SI section).

A comparison of the chemical profiles of the chromatograms

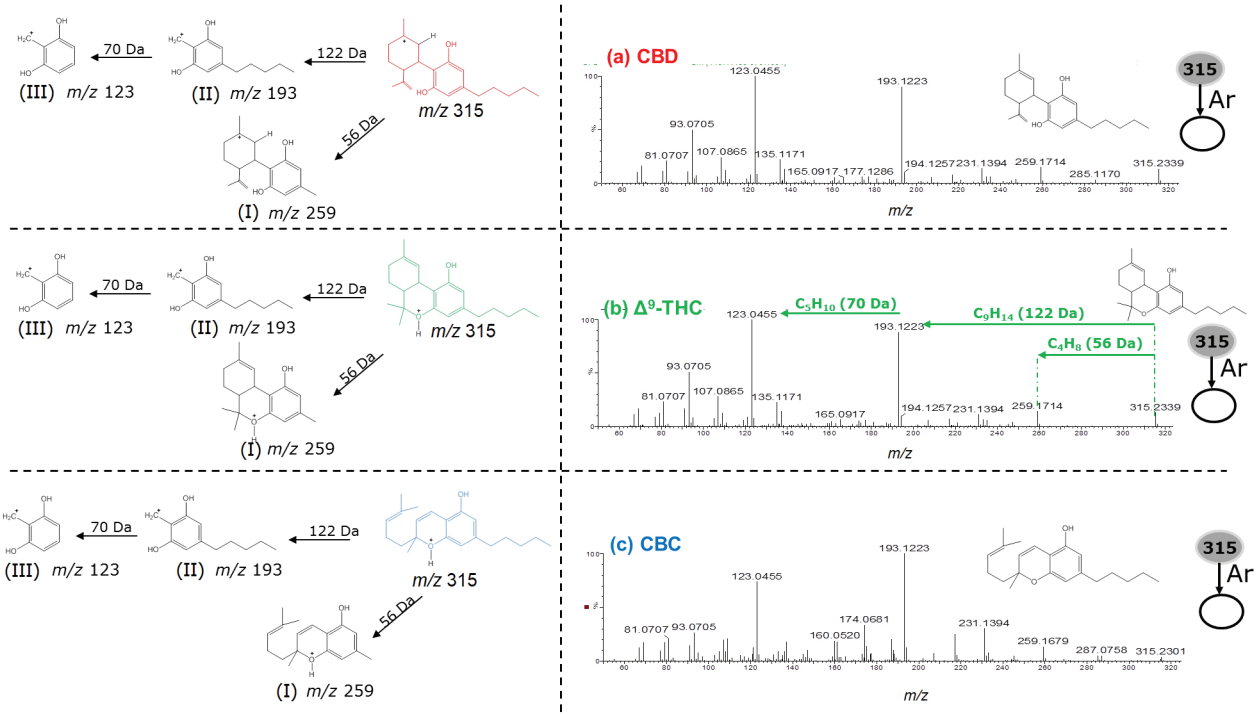

Figure 4. CID experiments of the constitutional isomers of *m/z* 315 ((a) CBD; (b) Δ^9-THC; and (c) CBC) as well as the proposed fragmentation of the ions of *m/z* 259, 193 and 123.

of an isomeric equimolar mixture of Δ⁹-THC, CBD and CBC standards (Figure 5a) with marijuana (Figure 5b) and hashish samples (Figure 5c) is shown in Figures 5a-5c. The results were obtained by UPLC-ESI(+)-QTOF-MS for *m/z* 315 in full scan mode (Figure 5a) and in the XIC mode (Figures 5b and 5c). Comparing the chromatographic profile of Figure 5a (standards) with those of Figure 5c (hashish), a total of eight isomers of *m/z* 315.2354, i.e., seven constitutional isomers of Δ⁹-THC are detected at t_R = 2.58, 3.86, 4.99, 5.21, 5.71, 6.85, 8.95 and 9.42 min. The ions with retention times at 3.86, 5.21 and 5.71 min correspond to CBD, Δ⁹-THC and CBC, respectively. Among them, the Δ⁹-THC compound is the most abundant species present in the marijuana and hashish samples (Figures 5b and 5c).

Comparing the results obtained by UPLC-ESI(+)-QTOF-MS with GC × GC-QMS (Figure 2 and Table 1), a higher number of Δ⁹-THC isomers was detected by UPLC-ESI(+)-QTOF-MS (7 against 4). In addition, the retention times observed by GC × GC-QMS for this same sample suggest that the peaks at 2.58, 4.99, and 6.84 min correspond to the Δ⁸-THC, Δ⁹-*cis*-THC, and CBL isomers, respectively. However, the peaks with t_R = 8.95 and 9.42 min may correspond to dimers containing as a basic structure an isomer of *m/z* 315, because this region of the chromatogram typically concentrates ions with *m/z* > 600 (see Figures S4a-S4c, SI section).

UPLC-ESI(–)QTOF-MS, UPLC-ESI(–)TWIM-MS, and ESI(–)MS/MS

UPLC-QTOF-MS (Figures 6a-6c) and UPLC-TWIM-MS (Figures 6d-6f) were applied in the negative ionization mode for the two isomeric standards of acid cannabinoids (Δ⁹-THCA A and CBDA, Figures 6a and 6d, and 6b and 6e, respectively) and their equimolar mixture (Figures 6c and 6f). The standards were detected in the deprotonated form, $[M - H]^-$, with *m/z* 357.2104, in which the CBDA (t_R = 3.49 and 4.90 min, Figures 6a and 6d, respectively) is eluted in a shorter time than Δ⁹-THCA A (t_R = 5.51 and 6.80 min, Figures 6b and 6e, respectively). This behavior is analogous to that observed for these same species in their respective neutral forms (CBD and Δ⁹-THC), Figures 3a-3c.

For the UPLC-ESI(–)TWIM-MS (Synapt G2-S) system, besides the separation of the two isomers, an additional peak (6.90 min) in the chromatogram of Δ⁹-THCA A (Figure 6d) and in the equimolar mixture of standards (Figure 6f) was observed. The proximity between the two peaks (6.80 and 6.90 min), Figures 6d-6f, suggests the detection of an isomer of Δ⁹-THCA A, due to a natural process of isomerization of the cannabinoid, i.e., the interconversion of the Δ⁹-THCA A molecule to Δ⁸-THCA A (Figure S5b, SI section), where the structural difference between the isomers would be in the position of the double bond of the six-membered ring. Another possibility is the isomerization of Δ⁹-THCA A to Δ⁹-THCA B (Figure S5a, SI section), where, in this case, compounds "A" and "B" have the carboxyl group in R1 and R3, respectively.[52,53]

In addition to the Δ⁹-THCA A isomers, signals at t_R = 5.20, 6.20 and 6.40 min (Figures 6c, 6d and 6f, respectively) with *m/z* 353 near Δ⁹-THCA A were detected. This peak, with *m/z* 353, might represent the standard oxidation of Δ⁹-THCA A, generating the cannabinolic acid (CBNA), which is detected as a $[C_{22}H_{26}O_4 - H]^-$ ion. CBNA is a cannabinoid precursor of CBN that cannot be

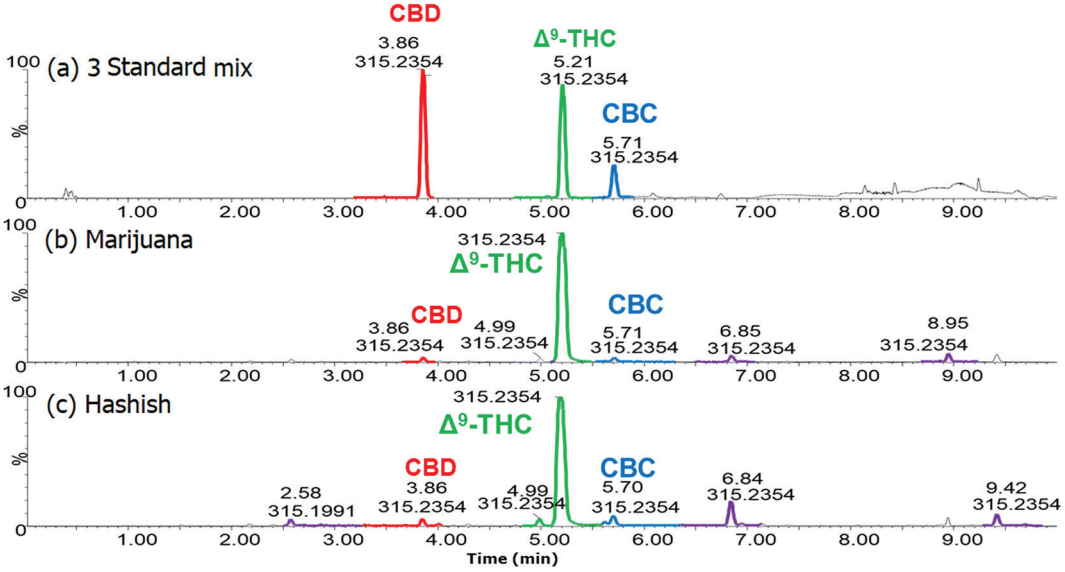

Figure 5. Chromatograms obtained by UPLC-ESI(+)QTOF-MS for an (a) isomeric mixture of Δ⁹-THC, CBD and CBC standards (full scan mode); and (b) marijuana and (c) hashish samples, both acquired in XIC mode for *m/z* 315.

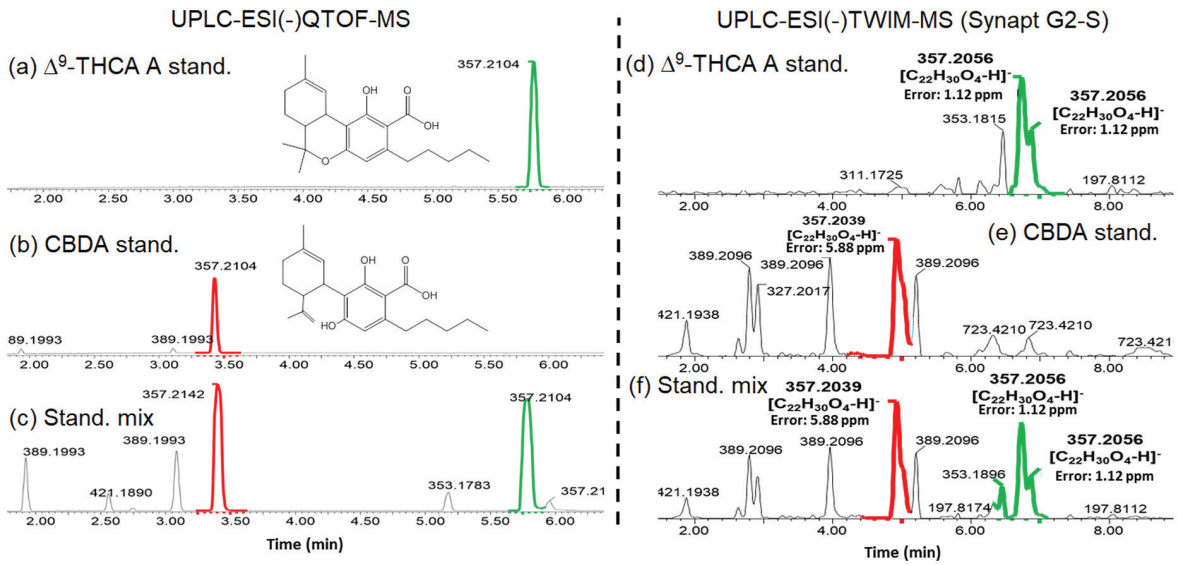

Figure 6. Chromatographic profile comparison between UPLC-ESI(–)QTOF MS and UPLC-ESI(–)TWIM-MS (Synapt G2-S) techniques for (a) and (d) Δ^9-THCA A; (b) and (e) CBDA standards; as well as (c) and (f) their equimolar mixture.

isolated from the fresh *Cannabis* plant. This compound can only be isolated from a *Cannabis* plant that was submitted to storage conditions or natural degradation.[3,53] Thus, the detection of the $[C_{22}H_{26}O_4 - H]^-$ ion at *m/z* 353 could be related to the conversion of the Δ^9-THCA A standard to CBNA.[16]

CID experiments were performed for the Δ^9-THCA A and CBDA standards, and the ESI(–)MS/MS results for *m/z* 357 are shown in Figures 7a and 7b. In contrast to the fragmentation profile observed for the molecules of Δ^9-THC and CBD (Figures 4a and 4b), the fragmentation profiles of *m/z* 357 were quite distinct. For the CBDA standard (Figure 7a), the major fragmentations are suggested in the *m/z* 357 → 339 transition, represented by the neutral loss of 18 Da (H_2O), resulting in the formation of the $[C_{22}H_{28}O_3 - H]^-$ fragment in structure (I) (Figure S6a, SI section). Subsequently, the *m/z* 339 → 311 transition, with the neutral loss of 28 Da (CO), generates the fragment proposed in structure (II), the $[C_{21}H_{28}O_2 - H]^-$ ion. Another possibility of fragmentation could be the *m/z* 357 → 313 transition, demonstrating the decarboxylation process, i.e., neutral loss of 44 Da (CO_2) resulting in the CBD molecule, which is present in the deprotonated form as the $[C_{21}H_{30}O_2 - H]^-$ ion (structure (IV)).

For the ESI(–)MS/MS results of the Δ^9-THCA A standard (Figure 7b), the *m/z* 357 → 313 transitions result in the main neutral loss of 44 Da (CO_2) and formation of Δ^9-THC,[5,15] resulting in the $[C_{21}H_{30}O_2 - H]^-$ ion,

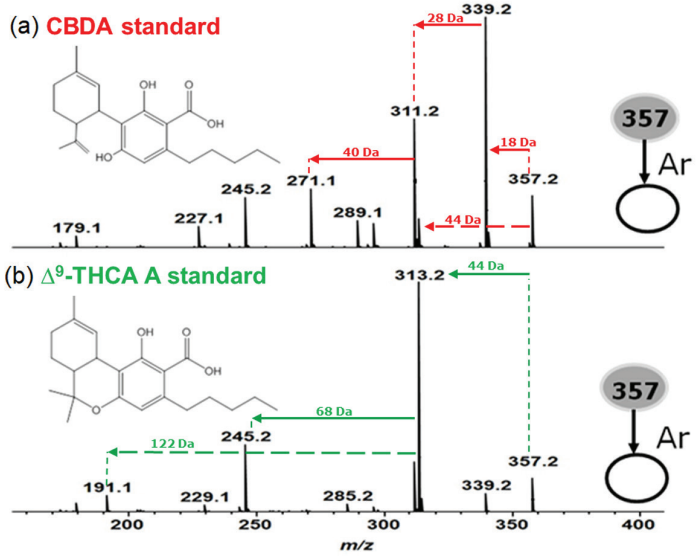

Figure 7. CID experiments for *m/z* 357 corresponding to the isomeric standards of (a) CBDA and (b) Δ^9-THCA A.

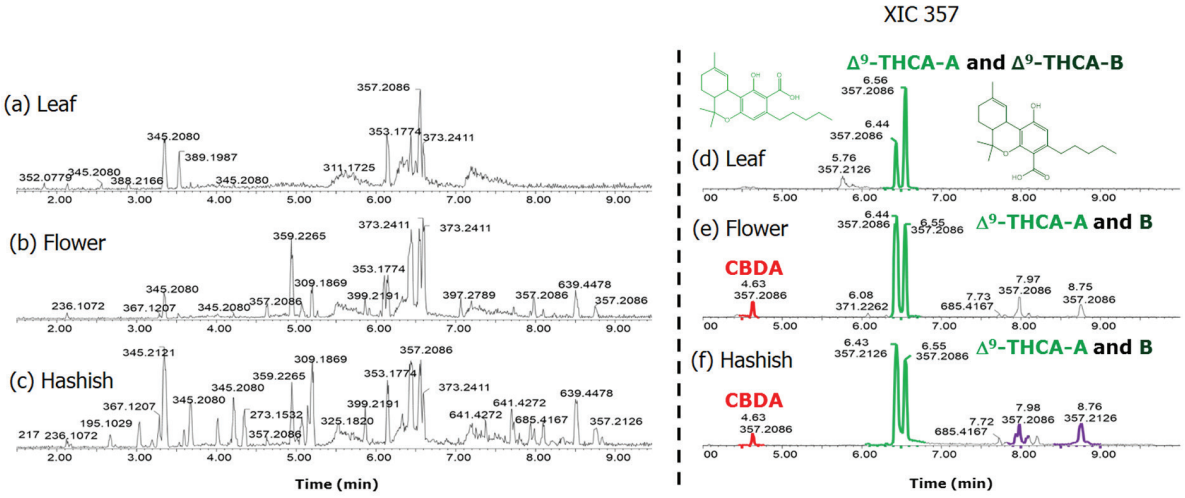

Figure 8. Chromatograms obtained by UPLC-ESI(–)TWIM-MS in full scan and XIC mode of *m/z* 357 for the (a) and (d) leaf; (b) and (e) flower; and (c) and (f) hashish samples.

structure (I). The subsequent transition, *m/z* 313 → 245, shows the neutral loss of 68 Da (C_5H_8) due to double homolytic cleavage from the six-membered ring, resulting in the formation of structure (II), $[C_{16}H_{22}O_2 - H]^-$. Another pathway of fragmentation from the *m/z* 313 ion is the neutral loss of 122 Da via carbon and oxygen bond cleavage (from the cyclic ether group), followed by the removal of the C_9H_{14} group, the *m/z* 313 → 191 transition, forming structure (III), $[C_{12}H_{16}O_2 - H]^-$. A proposed fragmentation mechanism for the molecules of CBDA and Δ^9-THCA A is suggested in Figure S6 (SI section).

Finally, parts of the *Cannabis* plant (leaf and flower) and of the hashish sample were analyzed by the UPLC-ESI(–)TWIM-MS technique in full scan mode (Figures 8a-8c) and XIC *m/z* 357 (Figures 8d-8f), in which a large number of compounds was primarily detected in the hashish and flower samples. Among the detected peaks, the signals of *m/z* 309.1869, 345.2080, 353.1774, 359.2265, 357.2086, 367.1207, and 373.2411 are highlighted and their assignments are shown in Table S1 (SI section).

For the isomers of *m/z* 357, five peaks were observed in the chromatogram (Figures 8e and 8f), corresponding to the samples of flower and hashish, respectively. They are: t_R = 4.63, 6.44, 6.55, 7.98 and 8.76 min. The peaks at 4.63, 6.43 and 6.55 min correspond to the CBDA, Δ^9-THCA A and Δ^8-THCA A or Δ^9-THCA B isomers, respectively. The other peaks at t_R = 7.98 and 8.76 min may correspond to dimers of CBDA and Δ^9-THCA A, as well as other isomeric forms of M_w = 358 Da, which are described in Figure 1c.

Conclusions

The GC-MS results demonstrated close retention times (Δt_R = 1.303 min) in separation of the five cannabinoids standards (Δ^9-THC, CBD, CBC, CBN and CBG), whereas GC × GC-QMS provided a substantially better identification and distinction of constitutional isomers of cannabinoids, where a total of 11 cannabinoids (Δ^8-THC, Δ^9-THCV, CBD, CBV, CBC, CBL, CBCR, Δ^9-THC, CBG, CBN, and 8α-OH-Δ^9-THC) were identified in the hashish sample. Among the cannabinoids detected, four are isomers of Δ^9-THC (Δ^8-THC, CBD, CBC, and CBL). On the other hand, complete chemical information was obtained by UPLC-ESI(–)QTOF MS and UPLC-ESI-TWIM-MS data, in which ESI(+) revealed the presence of seven constitutional isomers of Δ^9-THC, whereas ESI(–) proved the presence of four isomers of Δ^9-THCA A.

Supplementary Information

Supplementary data (chromatograms and EI-MS spectra of Δ^9-THC, CBD, CBC, CBN and CBG by GC-MS; mechanism for the fragments of the isomeric standards (CBD, Δ^9-THC, and CBC) proposed in more detail; chromatograms obtained from the UPLC-ESI(+)-QTOF-MS analysis of standards isomeric mixture of Δ^9-THC, CBD and CBC; marijuana and hashish samples (acquired in the full scan mode); Δ^9-THCA A and B, and Δ^8-THCA A molecule; Δ^9-THCA A and CBDA standards (ESI(–)MS/MS) and fragmentations suggested in the main transitions; and main detected peaks by UPLC-ESI(–)TWIM-MS highlighted and their assignments) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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