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Profiling of seized *Cannabis sativa* L. flowering tops by means of microwave-assisted hydro distillation and gas chromatography analyses

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ABSTRACT

This research aimed to support police forces in their battle against illicit drug trafficking by means of a multi-technique approach, based on gas chromatography. In detail, this study was focused on the profiling of volatile substances in narcotic Cannabis sativa L. flowering tops. For this purpose, the Scientific Investigation Department, RIS Carabinieri of Messina, provided 25 seized samples of Cannabis sativa L. The content of Δ^9-tetrahydrocannabinol (THC), useful to classify cannabis plant as hemp ($\leq$ 0.2 %) or as marijuana ($>$ 0.2 %), was investigated. Essential oils of illicit drug samples were extracted using a microwave-assisted hydro-distillation (MAHD) system; GC–MS and GC-FID analytical techniques were used for the characterization of the terpenes and terpenoids fingerprint. Furthermore, the enantiomeric and carbon isotopic ratios of selected chiral compounds were investigated using a heart-cutting multidimensional GC (MDGC) approach. The latter exploited a combination of an apolar column in the first dimension, and a chiral cyclodextrin-based column in the second one, prior to parallel isotope-ratio mass spectrometry (C-IRMS) and MS detection. Finally, all the data were gathered into a statistical model, to demonstrate the existence of useful parameters to be used for the classification of seized samples.

1. Introduction

Cannabis sativa L. is the most widely cultivated plant for illicit purposes worldwide, and it remains the most consumed illicit substance. In 2021, the amount of narcotic *Cannabis sativa* L. seized in Europe reached its highest level in the last decade, also due to the high availability of this drug [1]. Europe remains an important production zone, although some EU Member States are putting in place changes in cannabis regulation, especially for recreational use. For instance, in December 2021, Malta legislated for home growing and cannabis use in private, alongside non-profit communal growing clubs, for recreational purposes, while the Netherlands is piloting a model for a closed cannabis supply dedicated only to cannabis coffee shops [1]. Contrarily, in Italy, the sale of cannabis for recreational use is not permitted, but it can be used for industrial purposes, e.g. for foods, cosmetics, semi-finished products (such as fiber, powders, oils, or fuels), research activities by public or private institutions [2]. In any case, following law no. 242 of 2016 the content of Δ^9-tetrahydrocannabinol (THC) must be lower than 0.2 % in the biomass. On the other hand, in Italy, the use of *Cannabis sativa* L. in the medical context is allowed under the authorization of the Ministry of Health. The latter, in September 2021, specified that companies producing *Cannabis sativa* L. can supply only the pharmaceutical companies authorized by the Italian Medicines Agency (AIFA), to manufacture active pharmaceutical ingredients. In addition, the cultivation of certified seeds of *Cannabis sativa* L. is authorized by European regulation when the THC content is below the legal limit (0.2 %).

The main illicit cannabis-based products are marijuana (herbal cannabis) and hashish (cannabis resin). The former is obtained mainly

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from the plant flowering tops together with some leaves [3], while the latter is produced through a relatively long cottage industry process consisting of successively drying, sieving, and finally pressing, or by means of butan hashish oil (BHO) extraction [4]. The illegal market represents one of the main concerns for European authorities, considering that in 2021, the EU Member States declared 202,000 seizures of hashish (equivalent to 816 tons) and 240,000 seizures of marijuana, amounting to 256 tons [1]. These data demonstrate the huge efforts put in by the police forces to identify and track drug trafficking organizations [5]. In this context, the characterization of seized drugs in terms of physical and/or chemical characteristics of illicit substances represents an increasingly useful tool to support drug-enforcement agencies. The Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) recommends the use of an analytical scheme based on validated methods for the correct identification of a drug or chemical [6]. This requires the use of multiple uncorrelated techniques, classified into three categories listed in order to decrease discriminating power from A to C, in which mass spectrometry is included as a Category A technique, and gas chromatography as a Category B technique. The guidelines describe the use of a Category A technique with at least one other technique (from either Category A, B or C), thus a GC–MS approach could be considered able to support law enforcement efforts to assist police work. Moreover, as already stated in previous research studies [7], the employment of MS detectors can be a useful tool to investigate, and group, seized samples, with the aim to track their provenance. Alongside cannabinoids content, cannabis inflorescences are also rich in terpenes and terpenoids, which largely contribute to the typical flavor of this plant [8].

The present study deals with the cannabinoids and terpenes profiling of 25 seized samples consisting of *Cannabis sativa* L. flowering tops confiscated from January 2022 to July 2023. Considering the inaccessibility of illicit cannabis samples, the research was carried out in collaboration with the Scientific Investigation Department RIS Carabinieri of Messina, which provided the distilled essential oils (EOs) of the seized samples. All the samples were first evaluated in terms of THC content, while the terpene profile was assessed by GC–MS and GC-FID analyses after microwave-assisted hydro-distillation (MAHD) extraction.

Lastly, a heart-cutting multidimensional gas chromatography (MDGC) approach was implemented, based on the combination of apolar and chiral stationary phases in the first and second dimension, respectively. The system was coupled in parallel with isotope-ratio mass spectrometry (C-IRMS) and MS detection, for studying the enantiomeric and carbon isotopic ratios of selected enantiomeric couples.

2. Materials and methods

2.1. Sample and chemicals

Twenty-five seized samples of *Cannabis sativa* L. flowering tops were provided by the Scientific Investigation Department RIS, Carabinieri of Messina (Messina, Italy). Ultrapure water was obtained from a Milli-Q advantage A10 system (Millipore, Bedford, MA, USA). n-Heptane (suitable for HPLC, ≥99 %) was purchased from Merck Life Science (Darmstadt, Germany). Analytical standards of *n*-nonane (C_9H_{20}) and *n*-nonadecane ($C_{19}H_{40}$) hydrocarbons were acquired from Merck Life Science and used as internal standards (ISTDs) for quantitative purposes. For linear retention index (LRI) calculation, a homologous series of C_7–C_{40} saturated alkanes was purchased from Merck Life Science. For cannabinoids determination, THC and cannabidiol (CBD) standards were purchased from Lipomed (Lipomed AG, United States), and androst-4-ene-3,17-dione was purchased from Steraloids (Steraloids Inc., United States). Methanol was purchased from Honeywell (Honeywell, United States). For the calibration of the $\delta^{13}C$ values concerning the Vienna PeeDee Belemnite (VPDB) scale, three certified alkanes from Indiana mix A7 were employed, viz. hexadecane ($\delta^{13}C$ −26.15 ‰), nonadecane ($\delta^{13}C$- 31.99 ‰) and eicosane ($\delta^{13}C$ −40.91 ‰) (Indiana University, Bloomington, IN).

2.2. Cannabinoids extraction from sample flowering tops and GC-FID analysis

For the determination of the THC and CBD content in the seized samples, the results correspond to the sum of CBD+CBD-A (acid form) and of THC+THC-A (acid form) in the top flowers, accounting for decarboxylation at the injection port. The samples were analysed following the analytical protocol of the U.N.O.D.C guidelines (United Nations Office on Drugs and Crime) was followed [9]. This method is suitable for cannabis samples whose THC content varies between 1 % and 30% *w/w*, allowing the identification of cannabinols and cannabidiols. The quantitative determination of THC was based on a preliminary extraction using an ISTD solution, namely androst-4-ene-3, 17-dione, in methanol. After, the extract was analyzed through GC-FID. Quantification was achieved based on the ratio between the analyte areas, compared to the ISTD, using a six-point calibration curve. In detail, 30–40 mg of homogenized sample were transferred into a tube containing 5 mL of a 0.5 mg mL^{-1} solution of ISTD. The samples were sonicated for 20 min, filtered through a 0.25 μm Teflon filter, and transferred into GC vials.

Quantitative analysis was carried out by means of a GC Agilent 6090 N (Agilent Technologies, United States), equipped with a MPS2 Gerstel autosampler (GERSTEL GmbH & Co.KG, Germany), and coupled to the FID. The capillary column was an HP-5, 15 m × 0.25 mm ID × 0.25 μm d_f from Agilent Technologies. The separation was achieved in isothermal conditions at 240 °C for 8 min. The injection volume was 1 μL in split mode (1:50) with an injector temperature of 290 °C. Helium was used as carrier gas at a flow of 1.0 mL min^{-1}. The FID temperature was set at 300 °C; the gas flows were: 35.0 mL min^{-1} for H_2, 390.0 mL min^{-1} for air, 15.0 mL min^{-1} for the make-up gas (He). GC-FID data were acquired and processed by the GC Chemstation REV B.04.03 software (Agilent Technologies, United States).

The calibration curves for THC and CBD were obtained through standard solutions, prepared at six concentration levels as follows: 0.1, 0.3, 0.5, 0.7, 0.9, and 1.0 mg mL^{-1}, each in a solution containing 0.5 mg mL^{-1} of androst-4-ene-3,17-dione in methanol. The coefficient of determination (R^2) was always ≥ 0.9999.

2.3. Microwave-assisted hydro-distillation of sample flowering tops

Cannabis sativa L. flowering tops were extracted by MAHD according to the conditions reported by Micalizzi et al. [8]. In detail, 80 g of the flowering tops were rehydrated using ultrapure water with a 1:3 vegetable-mass/water ratio. After mixing and soaking, the vegetable mass was transferred in a 2 L DRY-DIST glass reactor of a Milestone "DRY DIST" (Milestone, Sorisole, Italy): each sample was distilled at 620 W for 30 min. A Clevenger-type apparatus, installed outside the microwave system, was provided by a vertical condenser with circulating ethylene glycol maintained at −5 °C through an external chiller. The extracted compounds were condensed in the Clevenger-type apparatus and the resulting EO was collected from the upper layer of the biphasic system into a collection vial.

Before GC analyses, 200 μL of ISTDs solution (1000 mg L^{-1} in *n*-heptane) and 800 μL of *n*-heptane were added to 10 mg of cannabis EO (dil. 1:100). The sample was homogenized in a vortex mixer and analyzed through GC–MS and GC-FID in triplicate.

2.4. GC–MS and GC-FID analyses of the EOs

GC–MS analyses of cannabis EOs were carried out on a GC-2030 NEXIS coupled to a single quadrupole mass spectrometer (QP-2020 NX) (Shimadzu Europa, Germany). The GC system was equipped with a split-splitless injector and an AOC-20i autosampler. The separation of

cannabis EO components was performed using a 30 m × 0.25 mm ID × 0.25 μm d_f SLB-5 ms (Merck Life Science) silphenylene polymer, virtually equivalent in polarity to a poly 5 % diphenyl/95 % dimethyl siloxane phase. The temperature program was as follows: 50 °C to 350 °C at 3 °C min^{-1}. The injection volume was 1.0 μL with a split ratio of 10:1 at 280 °C. Helium was used as carrier gas at a constant linear velocity of 30 cm s^{-1}. The MS system was operated in scan mode in the range of 40–550 *m/z*. Ion source and interface temperatures were 220 °C and 250 °C, respectively. GC–MS chromatograms were acquired and processed using the GCMSsolution software *ver.* 4.50 (Shimadzu). Peak identification was accomplished by exploiting the FFNSC 4.0 mass spectral library (Shimadzu), using two different identification criteria: mass spectral similarity (≥ 85 %) and linear retention index (LRI) tolerance window (± 5 units).

The GC-FID analyses were carried out using a GC-2030 NEXIS (Shimadzu) equipped with a split/splitless injector, an AOC-20i autosampler, and coupled to FID. All the parameters as well as the analytical column were the same as previously described for the GC–MS analyses. GC-FID chromatograms were acquired and processed by the LabSolution software *ver.* 5.93 (Shimadzu). Quantitative results were expressed as mg g^{-1} of EO, by using the ISTD method. For this purpose, FID response factors (RFs) were applied for each chemical class according to Costa et al. [10] as follows: monoterpenes and sesquiterpenes aliphatic hydrocarbons, RF = 1.0; oxygenated terpenes grouped in aldehyde, ketone, and alcohol classes, RF = 1.3; epoxides class, RFs = 1.5; esters class, RF =1.6. As known, the response of a FID to a hydrocarbon is proportional to the number of oxidizable carbon atoms, subsequently ionizable, present in the molecule. The use of RFs becomes necessary because, in the case of oxygenated compounds, there are already oxidized carbon atoms that are no longer ionizable, resulting in a reduced response from the FID [11].

2.5. Enantio-selective MDGC—C-IRMS/qMS analyses of the cannabis EOs

Simultaneous chiral and isotopic analyses of the cannabis EOs were carried out through the prototype MDGC—C-IRMS/qMS system, already described elsewhere [12,13]. Briefly, the system consisted of two GC-2010 Plus (Shimadzu) defined as GC-1 and GC-2 connected through a Deans Switch (DS) transfer device, able to operate in stand-by and cut mode. In the standby mode, the eluent from the GC-1 was directed to an FID detector, connected to the DS device via a 0.25 m × 0.18 mm ID stainless steel uncoated column (Merck Life Science), and exploited for monitoring the eluent from the first dimension (^{1}D). In the cut mode, the eluent from the GC-2 was split to a single quadrupole mass spectrometer (QP-2010 Ultra, Shimadzu) and a VisION IRMS system through a T-union. In detail, the effluent was diverted to the IRMS system via a 0.7 m × 0.32 mm ID uncoated column (Merck Life Science), connected to a GC V furnace system (Elementar Analysensysteme GmbH) maintained at 850 °C, and in parallel to the qMS system via a 3.5 m × 0.1 mm ID uncoated column (Merck Life Science). The temperature of the ion source and interface were maintained at 200 °C and a mass range of 40–550 *m/z* was monitored at an acquisition speed of 10 Hz. Data were acquired by the GCMS solution software *ver.* 4.50 (Shimadzu). Compound identification was carried out by using the FFNSC 4.0 mass spectral library (Shimadzu), exploiting MS similarity (> 85 %) and LRI correspondence (± 5 units), as previously described. About the MDGC system, GC-1 was equipped with a split/splitless injector (280 °C) using a split ratio of 10:1. The ^{1}D column was an SLB-5 ms 30 m × 0.25 mm ID × 0.25 μm d_f (Merck Life Science) operated with a constant helium flow of 1 mL min^{-1}. Pressure and temperature programs were exploited according to Cucinotta et al. [12]. In detail, the pressure program was from 185 kPa (7 min) to 247 kPa (5 min) at 1.89 kPa min^{-1}, to 300 kPa at 1.89 kPa min^{-1}, and finally to 330 kPa at 9.50 kPa min^{-1}, while GC-1 temperature program was as follows: 50 °C (held for 7 min) to 150 °C at 3 °C min^{-1} (held for 5 min), then to 227 °C at 3 °C min^{-1}, and finally to 280 °C at 15 °C min^{-1}. The FID was maintained at 330 °C, supplied by 40 mL min^{-1} of H_2 and 400 mL min^{-1} of air. GC-2 was equipped with a 25 m × 0.25 mm ID × 0.25 μm d_f MEGA-DEX ASX chiral column (MEGA, Milano, Italy). The analyses were carried out according to the following temperature program: 40 °C (22 min) to 76 °C (5 min) at 2 C min^{-1}, to 145 °C at 3 °C min^{-1}, and finally to 195 °C at 8 °C min^{-1}. A pressure program was applied to an auxiliary pressure control (APC) to hold a constant carrier gas flow of 1 mL min^{-1} in the ^{2}D column: 140 kPa (22 min) to 165 kPa (5 min), to a final pressure of 210 kPa at 1.39 kPa min^{-1}. An auxiliary helium line, monitored through a second channel of the APC unit, was used in the furnace to allow proper control over the open split conditions for IRMS detection. On the IRMS side, the following settings were applied to an Elementar VisION system (Elementar Analysensysteme GmbH, Langenselbold, Germany): acceleration voltage: 3795.805 V; trap current, 600,000 μA; magnet current, 3,700, 000 mA. IRMS results were handled by the lyticOS stable isotope data processing software (Elementar Analysensysteme GmbH, Langenselbold, Germany). All the analyses were carried out in triplicate and the standard deviations for IRMS measurements were found to be < 0.5 ‰.

2.6. Multivariate data analysis

Multivariate statistical analyses were carried out to investigate possible correlations among the seized samples. The content in cannabinoids, the volatile quali-quantitative profile, as well as the enantiomeric and isotopic ratios of the main volatile terpenes were used for the statistical evaluation. In detail, with the support of R-studio software (package:stats), it was possible to conduct hierarchical agglomerative clustering [14]. For hierarchical cluster analysis, *ggplot2* and *factoextra* libraries were used. Euclidean distance was used as the distance metric, and the Ward's agglomerative method [15] was adopted as the clustering method, due to the high variability among the samples. The clustering result was visually represented by a dendrogram. Unsupervised exploratory data processing was completed applying principal component analysis (PCA) [16], with the aim of confirming the presence of sample groupings – by inspection of score patterns – and understanding relationships between samples and measured variables – by joint examination of scores and loadings. Prior to PCA, data were pre-processed by column autoscaling [17]. As well as for hierarchical cluster analysis, the *factoextra* library was used.

3. Results and discussion

3.1. THC and CBD contents in seized cannabis sativa L. samples

THC and CBD contents in the 25 seized samples were determined by means of GC-FID analysis. Due to the decarboxylation occurring in the injection port, CBD-A and THC-A forms were finally detected as CBD and THC. Cannabinol (CBN) was not detected in any sample: as an indicator of sample degradation, when detected together with oxidative compounds, this component provides valuable information about the age of the samples. Thus, the absence of CBN indicates the freshness and good conservation state of the seized samples. Table 1 summarizes the results obtained for all the analyzed samples, seized as marijuana (14 samples) or as plants (11 samples). It could be immediately concluded that all the seized samples were illicit, due to the significant amount of THC detected in each sample, ranging from 0.80 % to 16.90 %. In this concern, the highest THC content (>15 %) was found in samples 2, 3 and 20, while the lowest values were found in samples 13 and 17. Dealing with CBD content, an opposite behavior was highlighted: samples 13 and 17 registered the highest values of 6.40 % and 4.10 %, respectively. Differently, the other samples analyzed showed CBD values lower than 0.5 %, except for sample 5, where a higher value was found (1.32 %).

Table 1
THC and CBD contents in the 25 seized samples.

ID	Type	% THC	% CBD
1	Marijuana	3.70	0.20
2	Marijuana	16.90	0.20
3	Marijuana	16.60	0.20
4	Marijuana	5.80	0.20
5	Marijuana	8.20	1.32
6	Marijuana	12.00	0.30
7	Plant	11.10	0.01
8	Plant	9.50	0.20
9	Plant	3.20	0.05
10	Plant	4.20	0.05
11	Marijuana	4.70	0.06
12	Marijuana	7.30	0.09
13	Marijuana	1.00	6.40
14	Plant	9.10	0.02
15	Marijuana	11.20	0.04
16	Plant	7.20	0.40
17	Marijuana	0.80	4.10
18	Plant	5.70	0.08
19	Plant	8.30	0.30
20	Marijuana	15.80	0.07
21	Plant	9.20	0.03
22	Marijuana	9.10	0.10
23	Marijuana	7.70	0.50
24	Marijuana	10.00	0.50
25	Plant	6.70	0.08

3.2. MAHD extraction and GC analyses of the distilled Cannabis sativa L. EOs

Due to legal restrictions, the Scientific Investigation Department (RIS) of Messina itself obtained cannabinoid-free EOs from *Cannabis sativa* L., by an optimized distillation procedure. The MAHD conditions were optimized using hemp inflorescences (80 g) purchased from a local store, at fixed microwave power at 620 W, and varying two different parameters: the distillation time and the vegetable-mass/water (g mL^{-1}) ratio. The use of a microwave power of 620 W avoided the distillation of cannabinoids since higher microwave power would have resulted in the presence of cannabinoid compounds in the distilled cannabis EOs, as reported by Micalizzi et al. [8]. The effect of the distillation time on the % yields was evaluated by analyzing the amounts of oils obtained after 12 min, 30 min, and 50 min of sample irradiation. In general, the first drop (about 20 mg) of EO was obtained after 5 min of distillation, at 89 °C measured in the DRY-DIST glass reactor. After 12 min (temp. 91 °C), the first fraction of cannabis EO was collected from the Clevenger-type apparatus and weighed. The distillation yield (*w/w*) was 0.635 % (507.7 mg of hemp oil). The other two fractions were collected at 30 min (temp. 91 °C) and 50 min (temp. 92 °C), with distillation yields of 0.375 % (300.4 mg of hemp oil) and 0.227 % (181.3 mg of hemp oil), respectively. These results indicated that the cannabis EO production increased up to 12 min of distillation; after, the EO production decreased to a min% yield at 50 min, as illustrated in Figure S1.

About the effect of the vegetable-mass/water ratio, the soaking process affects the oil yield because the vegetable mass absorbs water, becoming swollen. This effect promotes the extraction and release of oil into the water during the MADH process [18]. Different volumes of water were used to rehydrate a mass of 80 g: 240 mL, 480 mL, and 960 mL. The experiments revealed the highest yield using 240 mL of soaking water, while a decrease of 10 and 20 % of the distilled oil was observed using 480 mL or 960 mL of water, respectively. This result agreed with previous data reported in the literature [19]. Operating at 620 W for 30 min with a 1:3 vegetable-mass/water ratio the distillation yields of the cannabinoid-free EOs obtained from *Cannabis sativa* L. ranged from 0.013 % to 1.250 %. The great variability in oil content could be related to numerous factors including cultivar/breed [8], geographical origin and environmental factors [20], cultivation techniques (e.g., indoor, and outdoor) [8], and growing conditions such as light intensity, irrigation, nutrients, and duration [9].

The volatile profile of the oils (Fig. 1) revealed a total of 120 compounds, belonging to different chemical classes including monoterpenes, sesquiterpenes, diterpenes, and aliphatic hydrocarbons as well as oxygenated compounds belonging to the aldehyde, alcohol, ester, ketone, and epoxide classes (Table S1). As for legal restrictions, cannabinoids were not present in all the EO samples thanks to the mild MAHD power conditions. Table S1 reported the concentration of each compound listed expressed in mg g^{-1} of EO, according to FID data. To highlight the differences between the different samples, Fig. 2 shows a heatmap of the terpene compounds in the 25 oils analyzed. The heatmap showed similar trends of terpenes in almost all samples analyzed indicating the phenotypic tract of the marijuana EOs. Specifically, the monoterpene fraction was mainly characterized by the presence of α-pinene, β-pinene, myrcene, limonene, linalool, fenchyl alcohol, and α-terpineol. Regarding the sesquiterpenes fraction, (E)-caryophyllene, γ-elemene, (E)-α-bergamotene, α-guaiene, (E)-β-farnesene, α-humulene, and selinene derivatives, including β-selinene, α-selinene, selina-4(15),7(11)-diene, and selina-3,7(11)-diene, were the most abundant compounds.

Finally, the GC analyses revealed the presence of some oxidation compounds such as caryophyllene oxide, humulene oxide, guaiol, and α-bisabolol. From the volatile point of view, the seized samples analyzed did not show significant differences with respect to hemp samples investigated by Micalizzi et al. [8].

3.3. Simultaneous multidimensional chiral gc and isotopic analysis

To deeply investigate the characteristics of the oils, a simultaneous enantiomeric and isotopic characterization was performed through an advanced chromatographic approach. As already demonstrated by our research group for hemp EOs [12], by exploiting MDGC in heart-cut mode. The selection and the transfer of the key terpenes from the first (^{1}D) apolar to the second (^{2}D) chiral stationary phase allowed for overcoming issues associated with co-elutions, before IRMS detection. In detail, α-pinene, β-pinene, limonene, linalool, α-terpineol, (E)-caryophyllene, selina-4(15),7(11)-diene, selina-3,7(11)-diene, and caryophyllene oxide were studied. With respect to previous reference on hemp [12], the data obtained in this study showed a different trend in terms of chiral forms. Table 2 summarizes the enantiomeric ratio of the main chiral terpenes analyzed.

In the seized samples, among the monoterpenes, a preponderance of the levorotatory form was observed for limonene and α-terpineol, while variable behaviours were registered for the same components in hemp [12]. On the other hand, a clear predominance was highlighted for the dextrorotatory form of α-pinene and β-pinene in hemp, while a variable behavior was registered in the seized samples.

About the sesquiterpene components, a slight predominance of the second eluting enantiomer was found for both selina-diene isomers in hemp [12], while a racemic behavior was almost always detected in the seized samples. The only two components having similar enantiomeric ratio between hemp [12] and seized samples were caryophyllene oxide and (E)-caryophyllene.

Fig. 3 shows the enantiomeric distribution among the monoterpene constituents in three of the samples analyzed (viz. samples 3, 6, and 14).

In detail, (-)-limonene, (+)-linalool, and (-)-α -terpineol were always predominant, although with variable relative amounts. Whilst β-pinene showed very variable ratios, being the (+) form predominant in 15 out of 25 samples, and the (-) form predominant in 6 samples. In the remaining 4 samples, racemic values were detected. As reported by Booth et al. for the analysis of narcotic samples, a variable enantiomeric distribution may be associated with a different expression of the terpene synthase genes, even more concerning genetic manipulation [21].

The (-) form of limonene showed an abundance >90 % in 12 samples, between 80 and 90 % in 8 samples, between 70 and 80 % in 4, and only one sample was below 70 %. Concerning α-terpineol, the (-) form

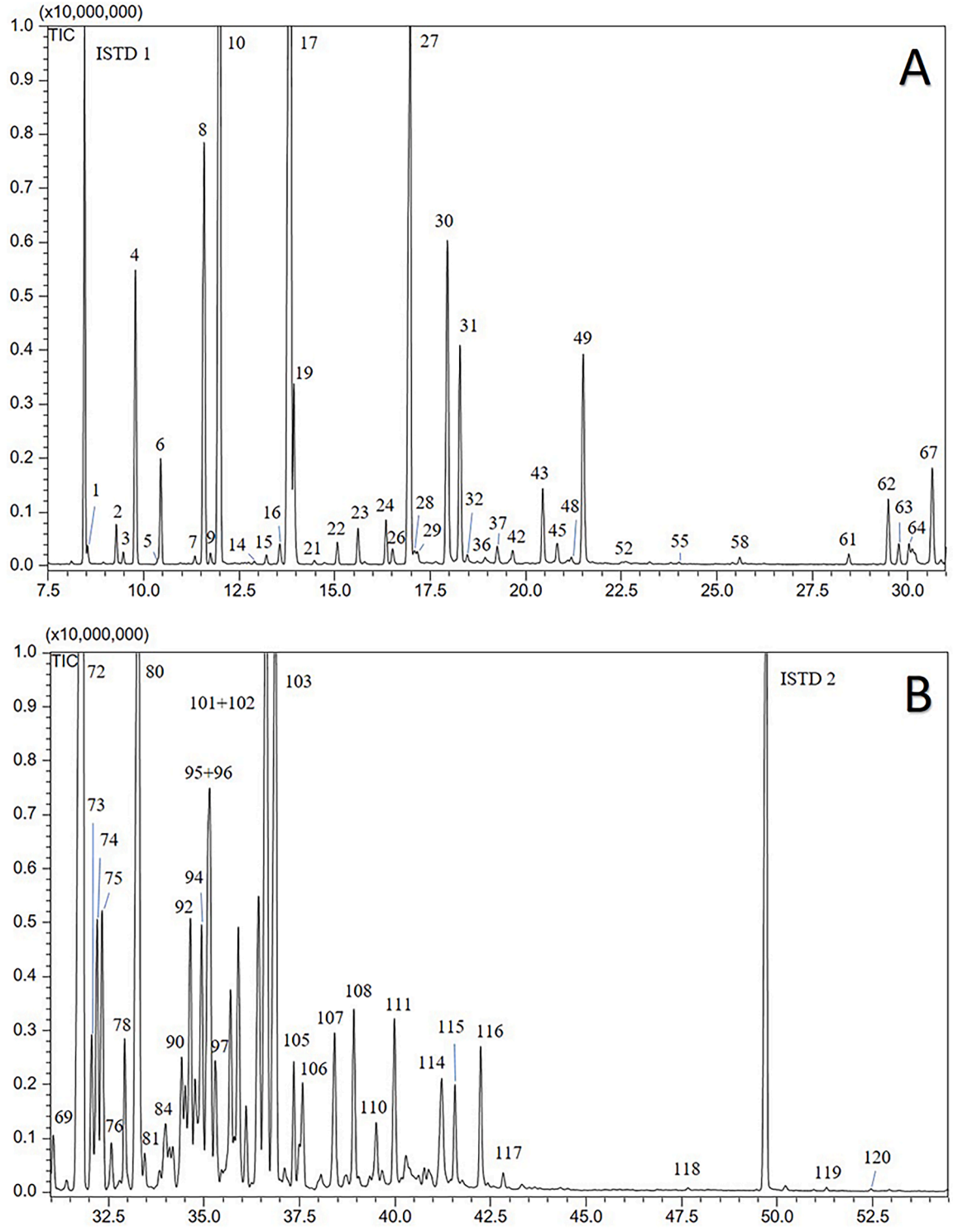

Fig. 1. GC–MS chromatograms of the monoterpene (A) and sesquiterpene (B) elution zones in the EO (sample n.7) distilled from Cannabis sativa L. flowering tops.

showed an abundance >90 % in 9 samples, between 79 and 90 % in 11 samples, and between 57 and 72 % in 5 samples.

Among the monoterpene compounds with a preponderance of the dextrorotatory form, in α-pinene the (+) form was >80 % in 18 samples, between 65 and 73 % in 5 samples, and only in two samples the ratio was inverted (sample 6, 3.6 % and sample 8, 24.2 %). A high consistency of the enantiomeric ratios was observed for the (+) form of linalool, with 24 out of 25 samples >87 %, and only one sample with a lower value (sample 25, 75.2 %).

Moving to the sesquiterpene compounds, as already highlighted for hemp EOs, an enantiomeric ratio always higher than 96 % was determined for the levorotatory form of caryophyllene oxide, while the (+) form of (E)-caryophyllene was not detected in any of the samples investigated [12]. Unlike what was observed in hemp EOs, both selina-diene isomers showed a racemic behavior, with only one exception being selina-3,7(11)-diene in sample 17.

About the carbon isotopic composition, Table 3 displays the $\delta^{13}C$ value of each terpene component investigated, producing a signal ≥0.5

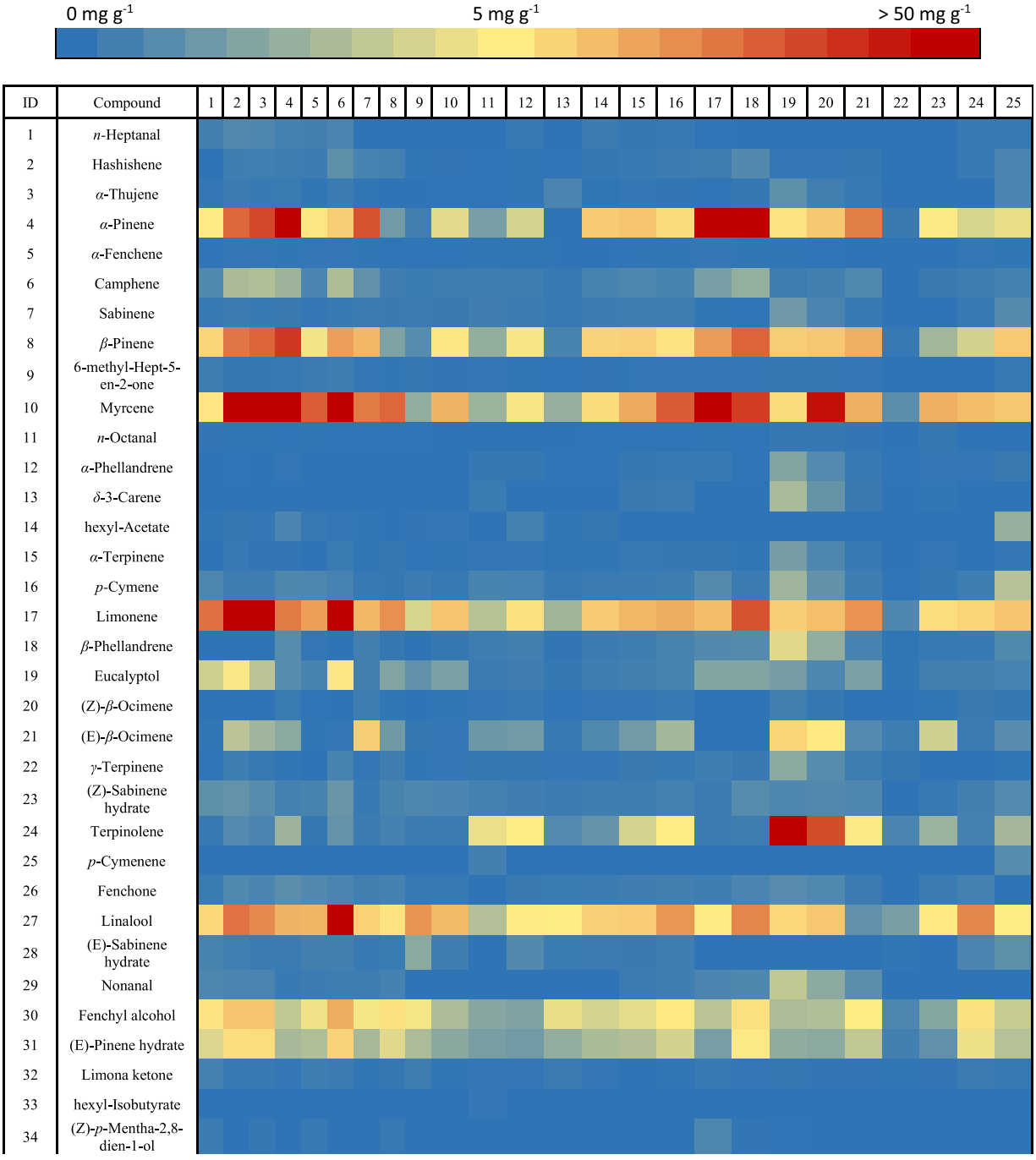

Fig. 2. Heat map of terpenes in seized Cannabis sativa L. distilled oils. Colors tending to red represent a higher concentration while a lower concentration is depicted by blue color.

nA to the IRMS detector. To the best of the author's knowledge, this paper describes for the first time in the literature the $\delta^{13}C$ values of terpene components in *Cannabis sativa* L. flowering tops.

In detail, a signal ≥0.5 nA was obtained for both enantiomers of α-pinene, β-pinene, limonene, linalool, and α-terpineol, together with (-)-(E)-caryophyllene and (-)-caryophyllene oxide. Even if the ^{2}D chiral stationary phase was able to separate the enantiomers of the two selina-diene isomers, the presence of a partial co-elution partially hampered the evaluation of the $\delta^{13}C$ value of the second eluting enantiomer of selina-4(15),7(11)-diene and the first eluting enantiomer of selina-3,7 (11)-diene. To guarantee a reliable measurement of the $\delta^{13}C$ value, all the target components should be resolved after the ^{2}D. In this concern, although a minimal co-elution was involved for (-)-limonene with eucalyptol, as visible in Fig. 3 (green and black traces), the very low amount of the co-eluted component did not significantly affect the calculation of the $\delta^{13}C$ value, as already reported in the literature [22, 23].

As reported in Table 3, the values were found to be more negative compared to the isotopic data retrieved for hemp [12], and sometimes outside the range of C_3 plants (−22.0 to −34.0 ‰) [24]. In this concern, Booth et al. reported that high negative $\delta^{13}C$ values displayed by marijuana samples, analyzed in bulk, may be associated with harvesting conditions, as well as with the employment of additional CO_2 sources and/or fertilizers [25]. Carbon dioxide is often employed to increase

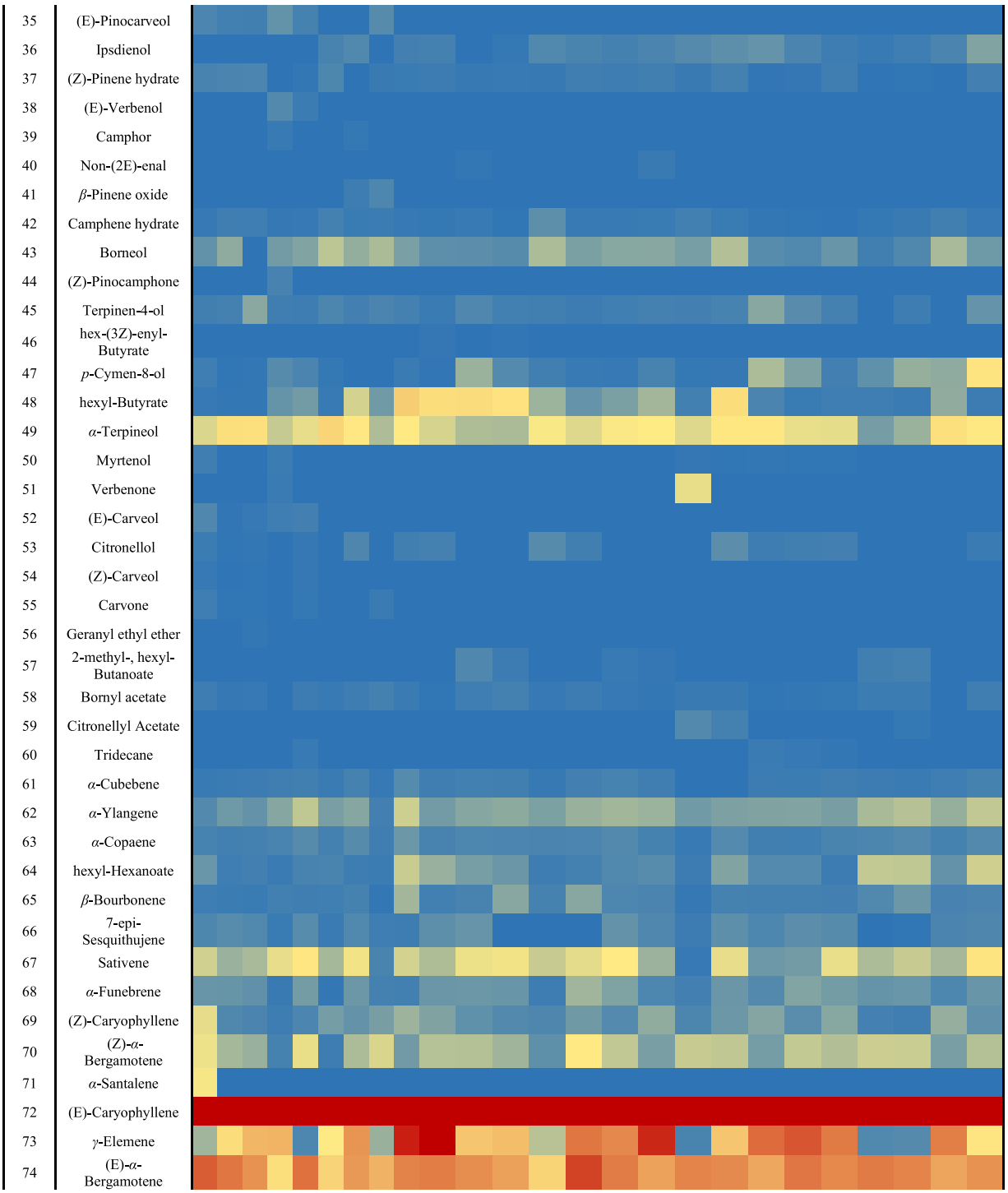

Fig. 2. (*continued*).

yields in enclosed spaces, such as greenhouses, in addition to atmospheric CO_2. According to the photosynthetic cycle, this condition affects the resulting isotopic composition of the metabolites in plants, leading to more negative isotopic values. The most variable $\delta^{13}C$ values were observed for (-)-caryophyllene oxide, with values ranging from −29.0 ‰ to −45.1 ‰, and 18 out of 25 samples ≥−40.0 ‰. A more consistent trend was observed in the 25 samples analyzed for the other terpene compounds, with only a few variations outside a max/min value of about 10 ‰ units, for the same analyte. As already reported in previous research on lemon EO [13], the $\delta^{13}C$ values obtained for the enantiomers of the same chiral component are noteworthy: while in most of the 25 samples analyzed only small differences were measured, some samples showed a shift of 5–6 ‰ units between the two enantiomers, as for α-pinene (sample 17), β-pinene (sample 4), and especially limonene (samples 17, 21).

3.4. Data correlation and pattern recognition

Further studies were carried out attempting to find some correlations between the analytical data obtained for the samples analyzed, and the time and place of the sample seizure. Table S2 summarizes the information given by the RIS of Messina about the seizure location, time, and

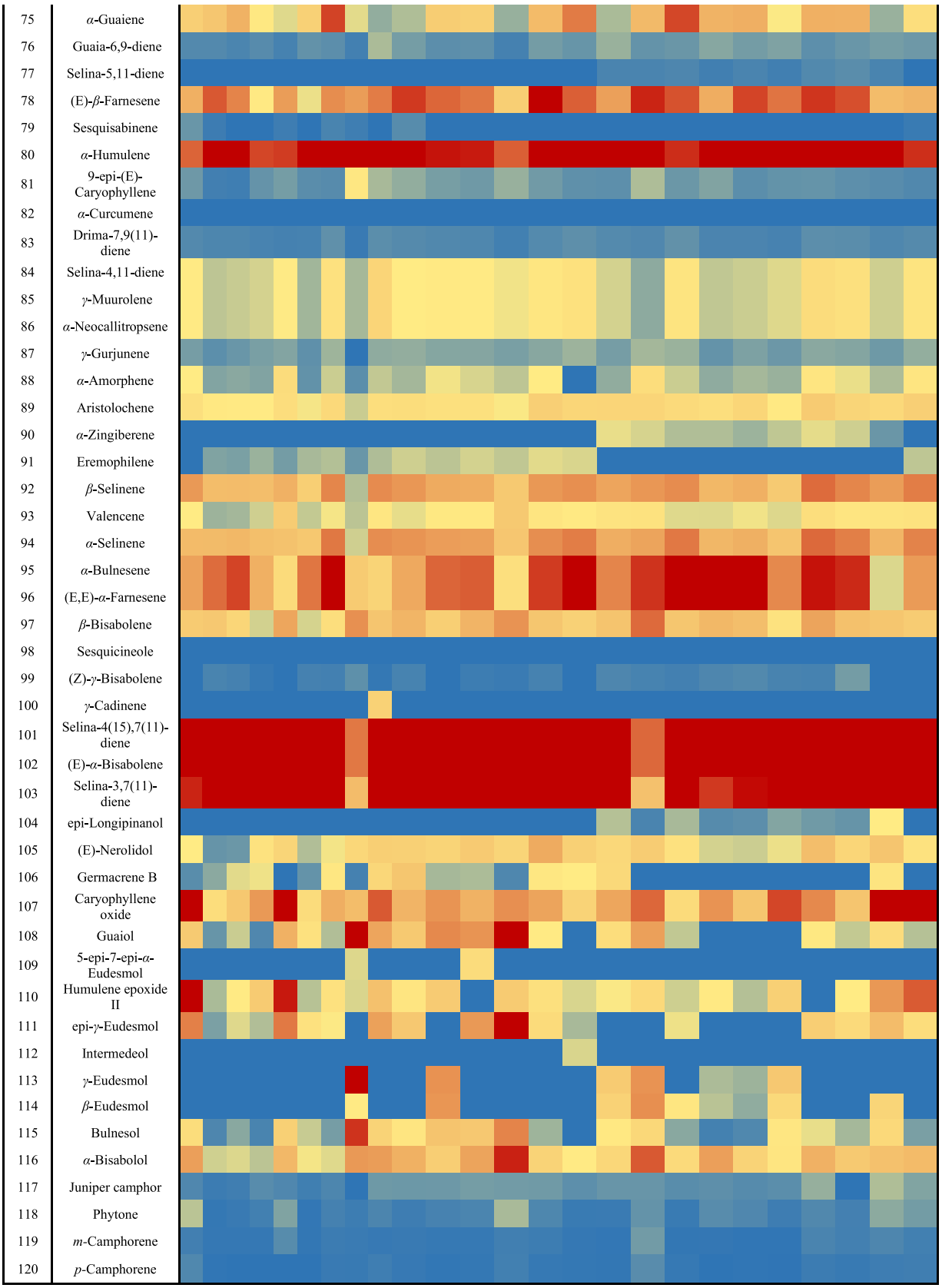

Fig. 2. (*continued*).

the type of seized samples (plant or marijuana).

Although no information was available about the Cannabis variety, the study aimed to highlight some critical traits along the seized samples, trying to track their origin and type. Consequently, the information retrieved by the different analytical approaches, including the cannabinoid content, quali-quantitative, chiral, and isotopic values of

Table 2
Enantiomeric ratios of the main chiral terpenes in the 25 seized distilled EOs analyzed. Levorotatory and dextrorotatory forms are reported according to the elution order on the chiral phase exploited [12].

Sample ID	α−Pinene		β-Pinene		Limonene		Linalool		α-Terpineol		(E)-Caryophyllene		Selina-4(15),7(11)-diene		Selina-3,7(11)-diene		Caryophyllene oxide	
	(-)	(+)	(+)	(-)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-/+)		(-/+)		(-)	(+)
1	31.8	68.2	31.6	68.4	96.0	4.0	3.2	96.8	92.1	7.9	100	0	50.2	49.8	51.7	48.3	98.4	1.6
2	31.8	68.2	30.4	69.6	95.7	4.3	2.0	98.0	94.5	5.5	100	0	49.6	50.4	50.3	49.7	96.3	3.7
3	26.7	73.3	35.0	65.0	95.8	4.2	1.5	98.5	94.2	5.8	100	0	49.8	50.2	50.5	49.5	97.6	2.4
4	4.3	95.7	74.2	25.8	87.4	12.6	8.3	91.7	81.8	18.2	100	0	50.6	49.4	50.9	49.1	97.7	2.3
5	30.3	69.7	34.5	65.5	94.8	5.2	11.1	88.9	90.2	9.8	100	0	49.1	50.9	49.7	50.3	97.3	2.7
6	96.4	3.6	0.3	99.7	96.1	3.9	2.4	97.6	94.5	5.5	100	0	49.9	50.1	50.5	49.5	96.2	3.8
7	6.4	93.6	64.1	35.9	90.9	9.1	7.0	93.0	90.4	9.6	100	0	50.2	49.8	51.2	48.8	98.2	1.8
8	75.8	24.2	9.1	90.9	95.4	4.6	5.0	95.0	90.7	9.3	100	0	48.8	51.2	50.7	49.3	97.0	3.0
9	14.5	85.5	59.2	40.8	86.4	13.6	4.3	95.7	91.2	8.8	100	0	49.1	50.9	50.3	49.7	98.3	1.7
10	11.6	88.4	61.6	38.4	92.4	7.6	4.9	95.1	91.5	8.5	100	0	49.3	50.7	50.2	49.8	98.2	1.8
11	10.1	89.9	70.4	29.6	77.7	22.3	12.0	88.0	62.3	37.7	100	0	49.5	50.5	50.5	49.5	98.6	1.4
12	10.3	89.7	66.4	33.6	86.3	13.7	6.4	93.6	72.5	27.5	100	0	49.5	50.5	50.5	49.5	98.1	1.9
13	17.9	82.1	57.2	42.8	86.4	13.6	6.1	93.9	86.2	13.8	100	0	48.5	51.5	49.0	51.0	97.6	2.4
14	6.7	93.3	67.4	32.6	88.7	11.3	11.0	89.0	87.4	12.6	100	0	49.8	50.2	50.5	49.5	97.8	2.2
15	11.2	88.8	52.7	47.3	91.8	8.2	9.4	90.6	86.8	13.2	100	0	50.0	50.0	50.8	49.2	96.6	3.4
16	13.1	86.9	49.9	50.1	91.1	8.9	6.4	93.6	85.8	14.2	100	0	48.6	51.4	48.7	51.3	97.5	2.5
17	4.1	95.9	87.0	13.0	76.7	23.3	9.2	90.8	81.5	18.5	100	0	43.4	56.6	36.8	63.2	97.5	2.5
18	7.9	92.1	63.7	36.3	91.7	8.3	7.7	92.3	89.6	10.4	100	0	50.1	49.9	50.9	49.1	98.3	1.7
19	16.9	83.1	66.3	33.7	67.5	32.5	9.4	90.6	57.1	42.9	100	0	48.8	51.2	47.0	53.0	97.6	2.4
20	9.3	90.7	66.3	33.7	80.2	19.8	6.0	94.0	67.0	33.0	100	0	49.3	50.7	47.6	52.4	97.7	2.4
21	7.9	92.1	69.2	30.8	92.6	7.4	11.7	88.3	86.6	13.4	100	0	50.0	50.0	50.3	49.7	97.7	2.4
22	8.6	91.4	70.1	29.9	76.6	23.4	13.0	87.0	79.6	20.4	100	0	50.0	50.0	50.2	49.8	94.4	5.6
23	11.5	88.5	63.3	36.7	76.8	23.2	11.5	88.5	82.8	17.2	100	0	49.5	50.5	50.0	50.0	94.4	5.6
24	13.4	86.6	47.8	52.2	88.9	11.1	5.8	94.2	87.3	12.7	100	0	48.5	51.5	48.6	51.4	97.7	2.3
25	34.9	65.1	50.2	49.8	89.0	11.0	24.8	75.2	60.1	39.9	100	0	49.9	50.1	50.2	49.8	97.5	2.5

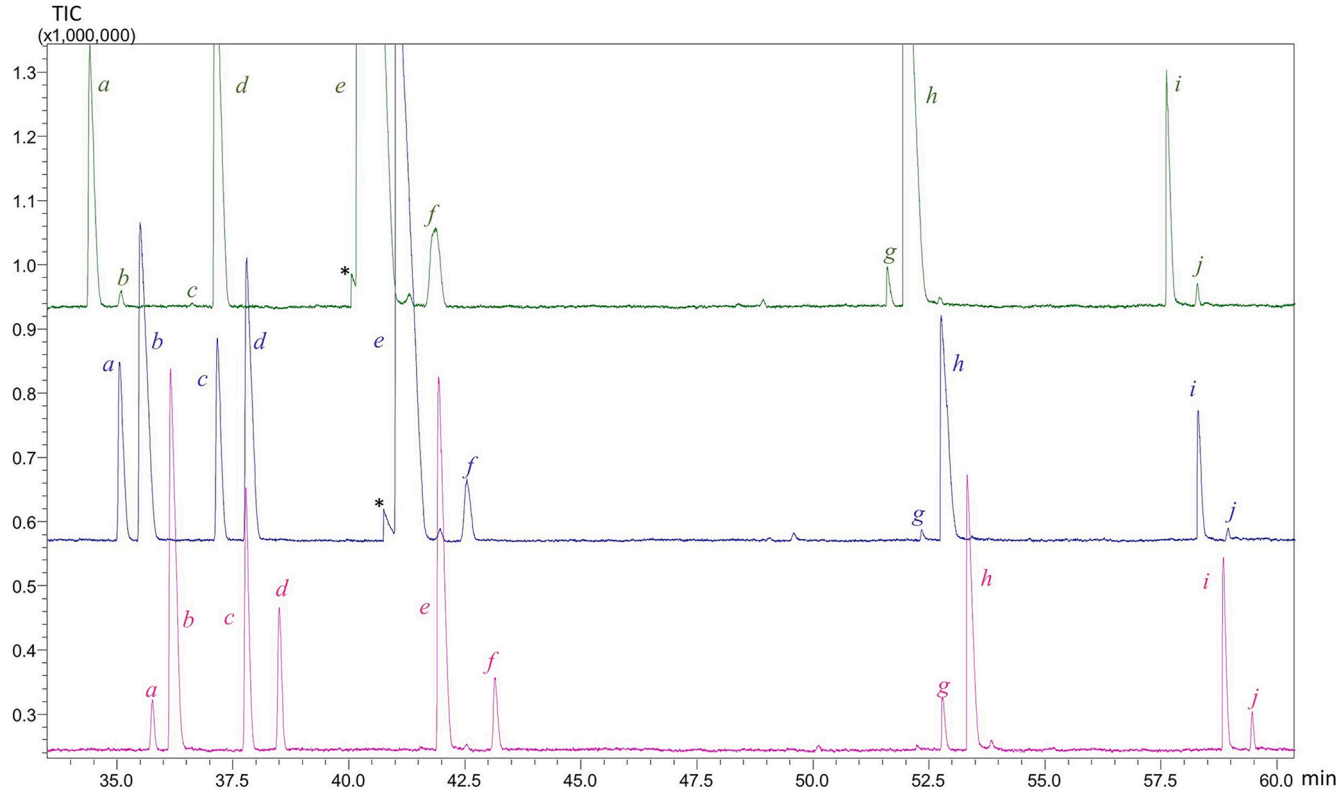

Fig. 3. Chromatograms of the heart-cut components on the 2D enantioselective stationary phase in of three distilled oils obtained from the seized samples: sample 6 (green trace), sample 3 (blue trace), sample 14 (pink trace). Peak IDs: a: (-)-α-pinene; b: (+)-α-pinene; c: (+)-β-pinene; d: (-)-β-pinene; e: (-)-limonene; f: (+)-limonene; g: (-)-linalool; h: (+)-linalool; i: (-)-α-terpineol; j: (+)-α-terpineol; *: eucalyptol. The chromatograms are shifted along the "x" axis for better visualization of shifts in the enantiomeric ratios.

Table 3
$\delta^{13}C$ value of the main terpenes (signal ≥ 0.5 nA) in the EOs distilled from the 25 samples. nd: not detected (< 0.5 nA). Since the elution order was not known for the two selina-diene isomers, they are reported as first 1st (-/+) and second 2nd (-/+).

SampleID	α-Pinene		β-Pinene		Limonene		Linalool		α-Terpineol		(E)-Caryophyllene	Selina-4 (15),7 (11)-diene 1st (-/+)	Selina-3,7(11)-diene 2nd (-/+)	Caryophyllene oxide
$\delta^{13}C$	(-)	(+)	(+)	(-)	(-)	(+)	(-)	(+)	(-)	(+)	(-)			(-)
1	−36.1	−33.8	−33.3	−35.6	−35.2	−34.1	nd	−34.0	−35.0	nd	−28.3	−33.7	−30.8	−34.4
2	−34.1	−33.1	−32.4	−33.6	−32.8	−34.2	nd	−33.0	−34.4	nd	−31.5	−31.6	−31.2	−36.4
3	−34.3	−32.5	−32.1	−33.8	−32.7	−34.3	nd	−32.6	−34.2	nd	−30.9	−31.5	−30.7	−36.4
4	−30.9	−32.5	−32.6	−38.9	−32.3	−32.6	−32.9	−32.3	−34.4	nd	−30.1	−31.2	−30.1	−36.1
5	−34.3	−32.0	−30.2	−31.7	−33.0	nd	−31.9	−31.6	−33.1	nd	−26.7	−30.6	−28.1	−32.6
6	−34.2	nd	nd	−33.3	−33.0	−35.9	−32.3	−32.9	−34.4	nd	−31.1	−32.4	−31.5	−37.7
7	−34.8	−33.9	−33.0	−35.6	−34.2	nd	−33.4	−33.6	−35.6	−33.9	−31.6	−32.7	−31.4	−39.0
8	−36.5	nd	nd	−34.6	−36.2	nd	nd	−33.4	−35.6	nd	−32.3	−32.8	−36.1	−33.5
9	nd	nd	nd	nd	−32.8	nd	−32.6	−33.3	−34.6	−33.5	−31.4	−34.4	−32.5	−40.7
10	nd	−34.2	−32.9	−33.8	−34.5	nd	nd	−33.6	−35.1	nd	−32.0	−34.8	−32.5	−41.0
11	nd	−34.4	−32.1	nd	−33.9	nd	nd	−33.3	−34.9	−33.5	−30.7	−31.0	−30.2	−39.4
12	nd	−34.1	−32.8	−33.2	−34.5	nd	nd	−33.6	−35.2	nd	−31.0	−31.1	−30.2	−41.1
13	nd	−33.4	nd	nd	−33.3	nd	nd	−32.4	−34.7	−31.4	−29.5	−34.7	−34.2	−45.1
14	nd	−33.2	−32.0	−33.7	−34.5	nd	nd	−34.1	−36.2	nd	−31.1	−32.1	−31.3	−40.0
15	−34.3	−34.0	−33.1	−34.8	−34.7	−31.9	−33.4	−33.4	−35.7	−33.5	−32.1	−34.5	−33.6	−39.8
16	−35.0	−34.3	−34.4	−36.2	−35.6	−32.6	−33.6	−34.2	−35.9	−33.8	−32.6	−36.6	−36.1	−42.2
17	−40.5	−35.4	−33.2	−33.5	−35.9	−30.3	nd	−33.0	−34.9	nd	−32.3	−34.0	−34.2	−37.8
18	−31.7	−32.0	−32.3	nd	−33.6	−34.5	−33.1	−33.7	−35.0	nd	−31.1	−32.4	−31.4	−43.0
19	−30.8	−31.0	−30.0	−30.1	−30.9	nd	nd	−29.8	−31.7	−29.8	−28.9	−29.8	−30.3	−35.8
20	−32.1	−32.6	−31.4	−32.2	−32.9	nd	nd	−30.8	−32.5	−30.3	−29.8	−32.2	−33.0	−38.9
21	−36.5	−34.7	−33.9	−36.2	−36.9	−31.4	nd	−30.6	−34.6	−31.2	−31.2	−34.4	−33.0	−41.4
22	nd	nd	nd	nd	nd	nd	nd	−34.1	−32.5	nd	−28.1	−27.7	−27.5	−32.4
23	nd	−31.0	nd	nd	−31.3	nd	nd	−30.8	−32.0	nd	−28.0	−28.2	−29.2	−29.0
24	nd	−34.4	−32.3	−34.5	−34.6	nd	nd	−34.4	−36.7	nd	−31.0	−31.8	−33.1	−36.3
25	−34.2	−33.1	−31.7	−32.5	−33.1	−30.7	nd	−31.8	−33.5	−32.2	−29.4	−31.4	−30.4	−36.3

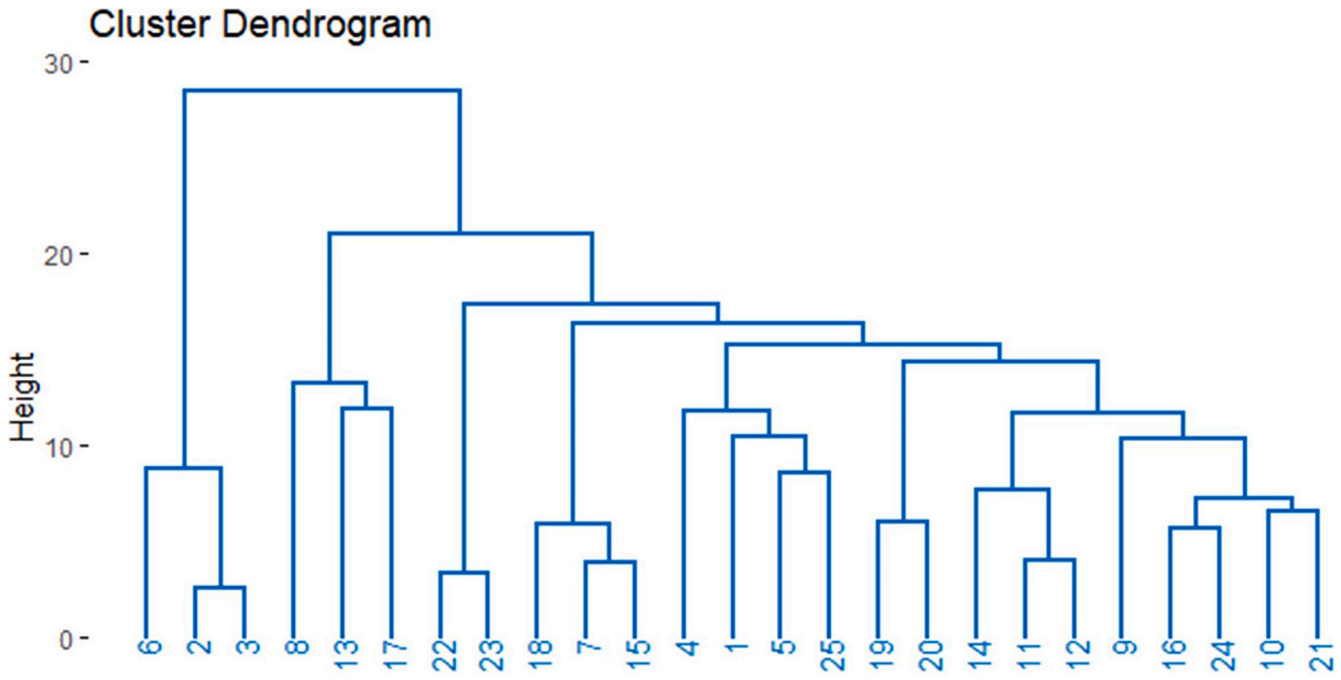

Fig. 4. Dendrogram graph based on the evaluation of the cannabinoids content, quali-quantitative profile, chiral and isotopic data for the 25 seized samples.

the volatile fraction, were thoroughly evaluated in a statistical approach.

In this concern, a hierarchical clustering dendrogram was built (Fig. 4), as reported in the Materials and Methods section.

The reduced height of the connecting branches suggested a close similarity between these samples in terms of cannabinoid content and quali-quantitative, chiral, and isotopic data. By comparing the hierarchical data and the general information in Table S2, it was possible to highlight some common traits among the samples of interest. In detail, the couples of major interests were samples 11–12, 22–23, 2–3, 19–20, and 7–15.

Looking in depth at the analytical data, samples 11–12 and 22–23, were characterized by higher concentrations of selina-4(15),7(11)-diene and selina-3,7(11)-diene. Such an outcome can be confirmed by inspecting the PCA biplot (Fig. 5).

In fact, samples 11–12 and 22–23 are located in the right bottom

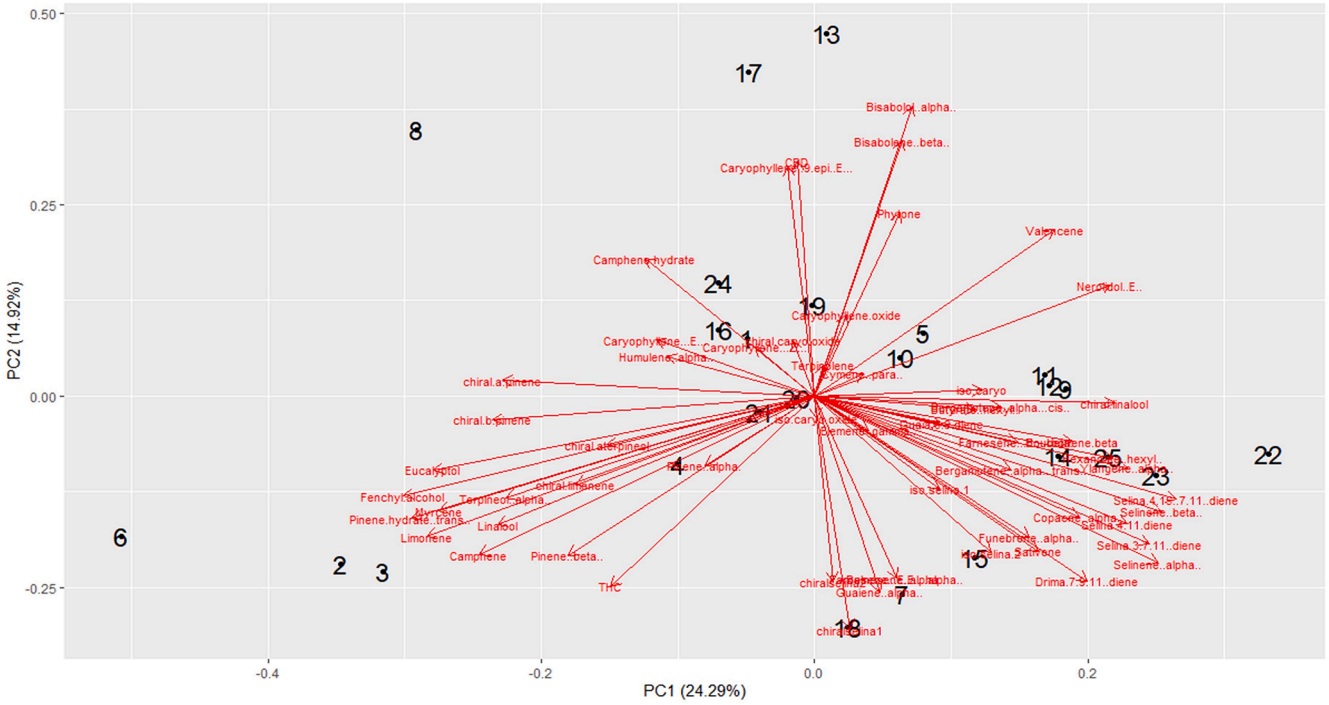

Fig. 5. PCA biplot: scores are represented by black numbers (sample ID), while loadings are indicated by red arrows and variable names.

corner (positive PC1 score values and negative PC2 score values), and the corresponding loadings (positive on PC1 and negative on PC2) do include selina-4(15),7(11)-diene and selina-3,7(11)-diene. On the contrary, samples 11–12 and 22–23 are characterized by lower concentration of camphene hydrate, whose loading is diametrically opposite. Regarding δ^{13}C values, the two pairs didn't show the same similarities observed for chiral data, because samples 22–23 were characterized generally by more positive δ^{13}C values. The volatile fractions of samples 2 and 3 were very similar, with a higher concentration of monoterpenes, such as myrcene, limonene, linalool, fenchyl alcohol, and (E)-pinene hydrate, concerning the other narcotic samples. This is fully confirmed by PCA results (Fig. 5), in which an evident correspondence is established, in the left bottom corner, between samples 2–3 and the variables listed above. It may also be deduced that samples 2–3 are characterized by a lower content of valencene and (E)-nerolidol, given the diametrically opposite location of the corresponding loadings in the biplot. Moreover, experimental data showed similar isotopic data, enantiomeric excesses, and THC content >16 %, being the samples with the highest THC content. Similar quali-quantitative values were observed in samples 19 and 20. Specifically, the most abundant compounds, i.e. (E)-caryophyllene, γ-elemene, α-guaiene and α-humulene showed comparable values among the two samples investigated. Regarding the enantiomeric data, the two samples also showed significant similarities, particularly for β-Pinene, α-terpineol, as well as for selina-4(15),7(11)-diene, selina-3,7(11)-diene and caryophyllene oxide. In this regard, despite being samples derived from different matrices and having different THC contents, their isotopic and chiral patterns, in conjunction with FID data, suggested a common origin.

About the information reported in Table S2, the samples mentioned above shared the same time and place of seizure, as well as the same peculiar traits, and could be then considered of the same type. The only exception was represented by the pair 19–20, differing in the sample type (plant and marijuana, respectively), suggesting the same origin. Two other samples paired in the dendrogram were samples 7–15. These similarities are confirmed by PCA outcomes (Fig. 5). Although the seizure time was different, the chiral, isotopic, and qualitative-quantitative data of both samples followed similar trends, suggesting a common origin.

The analytical patterns observed for the investigated samples were consistent with the information provided by the police (Table S2): samples 11–12 and samples 2–3 were seized in the same place, samples 22–23 belonged to the same plantation as well as samples 19 and 20, and samples 7–15 were seized in the same district.

4. Conclusions

This paper reports the development of a multi-technique approach to evaluate the possibility of tracing the origin of seized narcotic samples. Although no information was available on the Cannabis varieties, nor about the sample aging, the combination of the quali-quantitative (volatile fraction and THC/CBD contents), chiral, and isotopic data suggested a close correlation between specific narcotic samples. It was possible to tentatively group the narcotic samples with common experimental results, as a result of the same or similar seizure date, thus suggesting a common origin. Future studies will aim at the collection of a higher number of seized samples, as a more rigorous methodology to trace the narcotic flowering tops, to further support police investigations.

CRediT authorship contribution statement

Giuseppe Micalizzi: Conceptualization, Data curation, Investigation, Supervision, Writing – review & editing. **Lorenzo Cucinotta:** Formal analysis, Methodology, Investigation, Writing – review & editing. **Valentina Chiaia:** Formal analysis, Methodology, Investigation. **Filippo Alibrando:** Formal analysis, Methodology, Investigation. **Francesca Cannizzaro:** Formal analysis, Methodology, Investigation. **Gabriele Branca:** Formal analysis, Investigation. **Pietro Maida:** Data curation, Conceptualization, Supervision, Writing – review & editing. **Paolo Oliveri:** Data curation, Writing – review & editing. **Luigi Mondello:** Supervision, Visualization, Conceptualization. **Danilo Sciarrone:** Conceptualization, Supervision, Visualization, Methodology, Writing – review & editing.

Declaration of competing interest

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

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.464994.

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