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Pharmacognosy

Qualitative terpene profiling of *Cannabis* varieties cultivated for medical purposes

Perfil de terpenos de variedades de Cannabis *cultivadas para uso medicinal*

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Abstract

With the upcoming medical *Cannabis* regulation, quality control methods on raw material will be required. Besides testing for contaminants and potency, there are also pharmaceutical and forensic interests in the determination of the terpene profile in different strains of *Cannabis* as complementary identification methods. A simple non-destructive HS-SPME GC-MS method was used to identify the terpene content in twelve *Cannabis* samples, four of them were of the hemp type (Harle-tsu), seven from various marihuana types and one of the intermediate type. They all were previously analyzed by HPLC to determine the potency (THC and CBD content). Spectral library matching was used to identify the terpenes compounds. Thirty terpenes compounds were detected, nine of them were present in all *Cannabis* samples and used to find their terpene profile: α-pinene, β-pinene, β-myrcene, D-limonene, terpinolene, linalool, caryophyllene, α-bergamotene and humulene. Three of them, caryophyllene, α-pinene and β-myrcene were found as larger components in most of samples. A principal components analyses (PCA) was performed. The four hemp type samples showed two different profiles, two samples showed caryophyllene as main component and the others two with β-myrcene as such. The marihuana type samples showed wider profiles with no clear patterns at all, which is not surprising because of the low number of samples. The simple methodology shows viable to set the terpenes profile for analyses of raw *Cannabis* material. Suitability for differentiation between different sorts of types needs more studies, with increasing numbers of samples.

Key-words: *Cannabis*, chemical profile, GC-MS, HS-SPME, terpenes.

Resumo

A partir da iminente regulação da *Cannabis* para uso médico, métodos para o controle de qualidade desta matéria-prima serão necessários. Além de testes para contaminantes e potência, há também interesse farmacêutico e forense na determinação do perfil de terpenos nas diferentes variedades de *Cannabis* como método complementar de identificação. Um método HS-SPME GC-MS simples e não destrutivo foi usado para identificar o conteúdo de terpenoides em doze amostras de *Cannabis*, quatro delas do tipo cânhamo (Harle-tsu), sete diferentes do tipo maconha e uma do tipo intermédio. Todas foram previamente analisadas por HPLC para medir a potência através dos teores de THC e CBD. Comparação com a biblioteca de espectros foi usada para identificar os terpenos. Trinta compostos tipo terpeno foram detectados, nove deles estavam presentes em todas as amostras em níveis significativos, os quais foram usados para definir o perfil de terpenos: humuleno, cariofileno, D-limoneno, α-pineno, β-pineno, β-mirceno, linalool, terpinoleno e α-bergamoteno. Três deles, cariofileno, α-pineno e β-mirceno resultaram em maior quantidade na maioria das amostras. De acordo com

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a análise de componentes principais (PCA), as quatro amostras da variedade Harle-Tsu (perfil de cânhamo com THC/CBD < 1) apresentaram dois perfis diferentes, em duas amostras o componente majoritário foi o cariofileno, enquanto nas outras duas amostras o β-mirceno foi o componente majoritário. As amostras de maconha (THC/CBD > 1) mostraram perfis mais amplos, sem padrões claros que pode ser justificado pelo reduzido tamanho amostral. O método mostrou-se simples e viável para determinação do perfil de terpenos em matéria-prima vegetal de espécies do gênero *Cannabis*. Por outro lado, a atribuição de perfis químicos às variedades específicas necessita de mais estudos com maior tamanho amostral.
Palavras-chave: *Cannabis*, perfil químico, GC-MS, HS-SPME, terpenos.

Introduction

The first report about *Cannabis* was addressed to the Chinese, who described its medicinal properties in the Chinese Pharmacopoeia Pen-Ts'ao Ching 2000 years ago. Assyrians considered *Cannabis* as the main drug of their Pharmacopoeia since 3000 years ago (Honório *et al.* 2006). The 1st Brazilian Pharmacopoeia (1929) describes the *Cannabis sativa* L. var. *indica*, with the generic name "maconha", "meconha", "diamba" and "cannabis", indicating the flowered summits as a raw material in preparing *Cannabis officinalis* extract or fluid extract of India hemp, hemp powder and the Indian hemp dye (Silva 1929).

The *Cannabis* genus is considered to be monospecific (*Cannabis sativa* L.), which is divided into several subspecies (*(Cannabis sativa* subsp. *indica* (Lamarck) Small & Cronquist, *Cannabis sativa* var. *kafiristanica* (Vavilov) Small & Cronquist, *Cannabis sativa* var. *ruderalis* (Janisch.) Liou) (UNODC 2009; IPNI 2020). However, the chemical and morphological distinctions by which *Cannabis* has been divided into these subspecies are often not readily discernible. It appears to be environmentally modifiable, and vary in a continuous pattern. For most purposes, it will suffice to apply the name *Cannabis sativa* to all cannabis plants encountered (UNODC 2009).

The main compounds of *Cannabis* are the cannabinoids (phenolic terpenes). The major cannabinoids are tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), and when both are converted to their neutral forms tetrahydrocannabinol (THC) and cannabidiol (CBD), respectively, it is observed the pharmacological effects. THC is psychoactive, with antiemetic and analgesic effects, while CBD is a depressor with antiseizure, anxiolytic and anti-inflammatory properties (Honório *et al*. 2006; Hill 2015).

Among non-cannabinoid compounds, more than 100 volatile substances were already found in *Cannabis*, contributing mainly to its unique aroma as well to the pharmacological effects (Pavlovic *et al*. 2018; Iseppi *et al*. 2019). According to Ross & ElSohly (1996) volatile oil from fresh buds is composed primarily of monoterpenes (92%) and sesquiterpenes (7%), but small amounts of alcohols, aldehydes, ketones and esters can be found as well (Pavlovic *et al*. 2018).

Cannabis terpenoids such as limonene, myrcene, α-pinene, linalool, β-caryophyllene, caryophyllene oxide, nerolidol and phytol among others share a common precursor with cannabinoids in biosynthetic pathways. Most of them are all flavor and fragrance components common to human diets that have been generally recognized as safe by the United States Food and Drug Administration (FDA) and other regulatory agencies (Russo 2011).

Cannabinoids and terpenoids are synthesized inside *Cannabis* glandular trichomes (Potter 2009). Monoterpenes usually predominate (limonene, myrcene, pinene), but these headspace volatiles do suffer diminished yields with drying and storage, resulting in a higher relative proportion of sesquiterpenoids (especially caryophyllene), as also often occurs in extracts (Ross & ElSohly 1996). Terpenoid production increases with light exposure, but decreases with soil fertility (Langenheim 1994). This is supported by the glasshouse experience that shows higher yields in plants with relative nitrogen deficiency just prior to harvest (Potter 2004), favoring floral over foliar growth. Volatile oils composition is more genetically than environmentally determined (Bassolé *et al*. 2010), despite vegetative propagation of high-performance plants under controlled environmental conditions (light, heat and humidity) (Potter 2009). Terpenoid components in concentrations above 0.05% are considered of pharmacological interest (Adams & Taylor 2010). Animal studies are certainly

supportive, mice exposed to terpenoid odors inhaled from ambient air for 1 h demonstrated profound effects on activity levels, suggesting a direct pharmacological effect on the brain, even at extremely low serum concentrations (*e.g*. linalool with 73% reduction in motility at 4.22 $ng \cdot mL^{-1}$, pinene 13.77% increase at trace concentration, terpineol 45% reduction at 4.7 $ng \cdot mL^{-1}$ (Buchbauer *et al*. 1993).

Selective cross-breeding of high-terpenoid- and high-phytocannabinoid-specific varieties are a rational target that may lead to improve the pharmacological approach to such disorders as treatment-resistant depression, anxiety, drug dependency and dermatological disorders, as well as industrial applications as safer pesticides and antiseptics. An interesting future with terpenoid *Cannabis* compounds may be achievable through further research of the entourage effect (synergistic effect).

The Brazilian Sanitary Agency (ANVISA) has authorized *Cannabis* extracts importation for medical purposes since 2015. Although the cultivation standards for drug research and production purpose are being formulated, the available *Cannabis* varieties in Brazil are still unknown or their studies are scarce in current scientific literature. The major difficulty to the authors lies in obtaining a sufficient number of samples of recognized origin, especially by legal reasons.

At Federal University of Rio de Janeiro (UFRJ), Brazil, an extension project, named Farmacannabis, was created to monitor the medical treatments with *Cannabis* products and to offer pharmaceutical support for patients or their parents who grow *Cannabis* and prepare their extracts with court authorization (Carvalho 2017). In order to study the chemical profile of *Cannabis* plants cultivated by individuals assisted by Farmacannabis project, a method by headspace solid-phase micro extraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS) was developed and the major volatile terpene profiles were identified to determine the differences among these plants and contributing for chemotaxonomic discrimination of the varieties of the species.

Material and Methods

Collection and sampling

Dried *Cannabis* flowers samples were obtained from plants cultivated by patients and patients' parents attended by Farmacannabis project. The growers reported the names of the cultivated varieties and the growing conditions. The samples were labeled in the laboratory by the reported names and by a code composed of letters and numbers (for example: SP001 means sample 1).

Twelve samples reported as being of the varieties Cinderella, Caetano Veloso, Harle-Tsu, Amnesia Haze, 24K Gold, Og Kush, Tolomelli and Cannatonic *Cannabis* were cultivated indoor and outdoor by cloning. According with report by growers the plants were kept under constant illumination, when they grew about 1m high they were kept in cycles of light and darkness, 12 h for each cycle, for flourish. The flowers were harvest when the glandular trichomes showed brown color. The time between the beginning of cultivation and harvest was between 4 and 6 months.

After the harvest, growers removed the leaves and dried the flowers between 7 and 15 days in well-ventilated area, protected from light and moisture. The *Cannabis* flowers were transported to laboratory by patients or their parents and storage at -20 °C until analysis is performed.

The sampling took place in the pharmaceutical support when individuals assisted by Farmacannabis requested information on the profile of the major cannabinoids, THC and CBD. Five cannabinoids, THC, THCA, CBD, CBDA and CBN were extracted from vegetable sample (100 mg) by organic solvents and quantified according De Backer *et al*. (2009) in a High Performance Liquid Chromatography system Thermo composed by quaternary pump model 600, type Rheos 5600, Accela autosampler and PDA Accela detector 20 Hz. Data were processed using the ChromQuest 5.0 software. The calibration curves were performed at concentrations between 1.00 and 30.00 mg/mL for all cannabinoids with certified reference standards THC, CBD, THCA, CBDA and CBN 1.0 mg/mL purchased from Cerilliant Corporation and diazepam (7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one; 99.9% purity) purchased from National Institute of Quality Control in Health, Fiocruz, Brazil was used as internal standard.

The total THC (the sum of THCA, THC and CBN) and total CBD (the sum of CBDA and CBD) were used to calculate the THC/CBD ratios and classified the specimen as hemp or marijuana according with the guidelines used by National Drug Analysis Laboratories of United Nations Office on Drugs and Crimes (UNODC, 2009) and by American Herbal Pharmacopoeia (AHP

2013). Marijuana shows THC/CBD ratio > 1 and hemp shows THC/CBD ratio < 1 (UNODC 2009). A distinction between "hemp" and "marijuana" is useful for patients to select the varieties of interest in seizures control (rich-CBD varieties) or in nausea and vomiting control in cancer patients (rich-THC varieties). The THC/CBD ratio around 1 is classified as "intermediate" (AHP 2013).

Terpenes extraction and analysis

Samples were homogenized by pulverization and 100 mg were introduced into a flat bottom 60 mL glass bottle with a rubber cap. The HS-SPME was performed with a 100% polydimethylsiloxane (PDMS) fiber Supelco, 100 µm coating thickness, recommended for volatile compounds. The bottle with the sample was heated in a ceramic hot plate with digital temperature control at 50 °C for 10 min. After the heating the SPME fiber was introduced and exposed into the head space bottle during 60 seconds and then the fiber was retracted and removed. Desorption procedure was done by injection in the GC-MS and exposed for 30 seconds in order to rich the full desorption of the volatile fraction (250 ºC temperature in the injection port) and in split mode (ratio 20:1).

Previously, blanks from the fiber and bottle were analyzed in order to identify any potential interference from used material sources. The GC-MS qualitative analyses were performed in a Shimadzu GCMS QP 2010 ultra system with 30 m x 0.25 mm × 0.25 µm 5% phenylmethylpolysiloxane capillary column (HP-5MS, Agilent), the oven temperature was set at 60 °C (5 min), increased from 20 °C min^{-1} to 170°C (3 min), increased from 20 °C min^{-1} to 260°C (2 min). Helium 5.0 analytical grade purity was used as carrier gas at a flow of 1.21 mL min^{-1}. Temperature applied were 250 °C for injector, 250 °C for interface and 230 °C for ion source. Data were acquired in the full scan mode (50-500 *m/z* mass range).

Terpenes identification was done using mass spectrum computerized databases (NIST11) and by the linear index of retention (LRI). The integration parameters were settled to the 30 compounds with highest Total Ion Current (TIC) abundance. Nine terpenes were present in all samples and were used to compare the profiles: α-pinene, β-pinene, β-myrcene, D-limonene, terpinolene, linalool, caryophyllene, α-bergamotene and humulene.

Prism 7.0 software was used to show the distribution of nine terpenes in samples and to make the descriptive statistics. The PAST program was used to perform the principal component analysis (PCA), the relative TIC percentages were calculated by considering the nine common terpenes and performed normalization between them totaling 100%.

Results and Discussion

Eleven samples donated by patients were from plants cultivated in Rio de Janeiro state and one sample was from plant cultivated in São Paulo. The geographical region, growing conditions, names of varieties reported by growers and THC/CBD ratios are showed in Table 1. Only the specimens reported as Harle-Tsu variety met the criteria for classification as hemp

Table 1 – Description of the *Cannabis* samples.

Variety	Grow condition	City (region)	Sample coding	THC/CBD ratio
Amnesia Haze	outdoor	Xerém	SP001	74.0
24K Gold	outdoor	Xerém	SP002	22.5
Cannatonic	outdoor	Paraty	SP003	9.8
Og Kush	indoor	Rio de Janeiro (North)	SP004	33.0
Cinderella	outdoor	Xerém	SP005	320.0
Caetano Veloso	indoor	Rio de Janeiro (South)	SP006	90.0
Tolomelli	indoor	Rio de Janeiro (North)	SP007	12.5
Tolomelli	indoor	Rio de Janeiro (North)	SP008	0.8
Harle-Tsu	indoor	Rio de Janeiro (South)	SP009	0.1
Harle-Tsu	outdoor	São Paulo (East)	SP010	0.1
Harle-Tsu	indoor	Rio de Janeiro (South)	SP011	0.1
Harle-Tsu	indoor	Rio de Janeiro (North)	SP012	0.1

according UNODC (2009) and American Herbal Pharmacopoeia (2013). The determination of THC/CBD ratios is necessary because hemp varieties are recommended for the manufacture of seizure control drugs.

The HS-SPME extraction and analytical method provided sufficient sensitivity to identify terpenes with abundance above 1×10^5 TIC. The Figure 1 shows the separation of 30 compounds and the Table 2 shows the identification of terpenes with abundance above 1×10^5 TIC in the chromatogram.

The HS-SPME extraction is a non destructive method and only 100 mg of sample is required for terpenes analysis by GC-MS and the same sample can be used to quantify cannabinoids by HPLC method.

Peak identifications were then assigned using MS spectral matching against reference spectra in the Wiley, NIST libraries and LRI. The identified terpenes were similar to those found previously in the analysis of dried *Cannabis* (Giese *et al*. 2015; Mariotti *et al*. 2016). A review article referred the monoterpenes myrcene, α-pinene, limonene and linalool and the sesquiterpenes β-caryophyllene, α-humulene, bisabolol and (E)-β-farnesene as the most common terpenes present in *Cannabis* varieties (Booth & Bohlmann 2019).

Nine terpenes (α-pinene, β-pinene, β-myrcene, D-limonene, terpinolene, linalool, caryophyllene, α-bergamotene and humulene) were present in all samples and could be used as complementary chemical markers in the qualification of the *Cannabis* genus plants. In order to compare the proportion of each nine terpenes in the samples, the relative levels were defined by the relative percentage TIC for each of nine terpenes in comparison with total percentage TIC (Fig. 2). The PCA analysis showed as main components β-myrcene, α-pinene, caryophyllene and terpinolene (Fig. 3). The Harle-Tsu samples showed two different profiles, two samples showed caryophyllene as main component and the others two presented β-myrcene as major component.

The varieties identified as Cannatonic, Tolomelli and Caetano Veloso showed more volatile monoterpenes, β-myrcene and β-pinene. The variety identified as Cinderella showed the highest level of terpinolene. The later eluting peaks consisted of sesquiterpenes, such as caryophylene, α-bergamotene and humulene. The predominance of these compounds could be due to this specific strain of *Cannabis*, or the nature of the sample tested, which was dried. The level of this compound should increase significantly in relation to other terpenes and terpenoids with drying. Consequently, the levels of the monoterpenes would be expected to be smaller, what was observed to some degree, especially in the hemp varieties (AHP 2013; Giese *et al*. 2015; Sterneson & Halpenny 2017). Among the monoterpenes and terpenoids the most abundant were α-pinene, β-pinene, β- mircene, D-limonene. The varieties identified as "Og Kush", "Cinderella" e "24K Gold" showed the lowest relative levels of α-pinene. Perhaps the relative low levels of monoterpenes in some samples can be attributed to drying conditions or even transport conditions to the laboratory.

The *Cannabis* samples analyzed in the present study were cultivated in different conditions

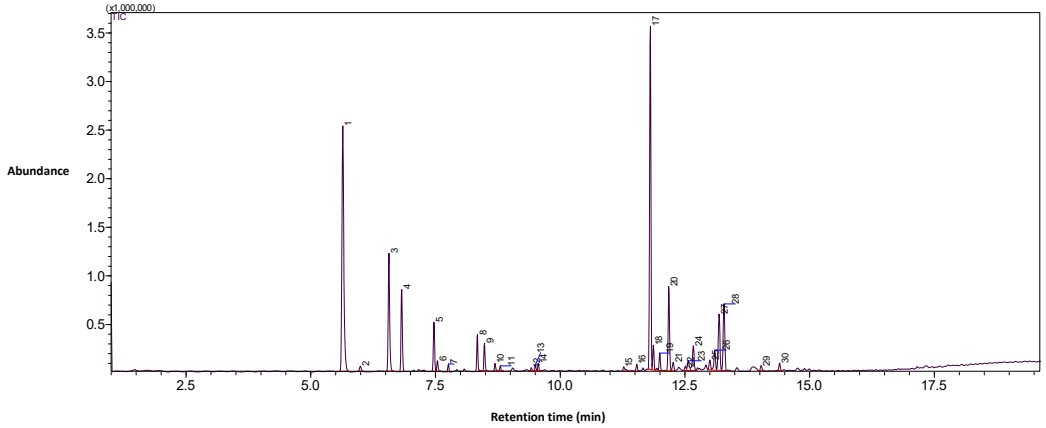
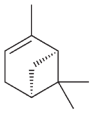
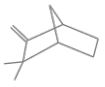
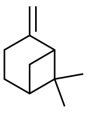
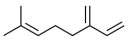
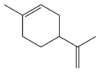
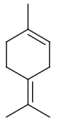
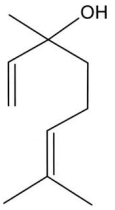
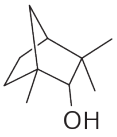
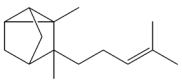
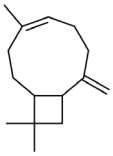
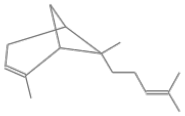
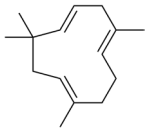
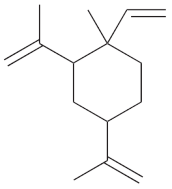
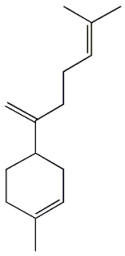
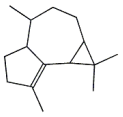
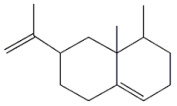
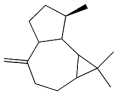
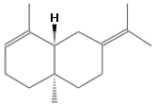
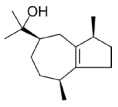
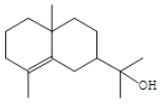


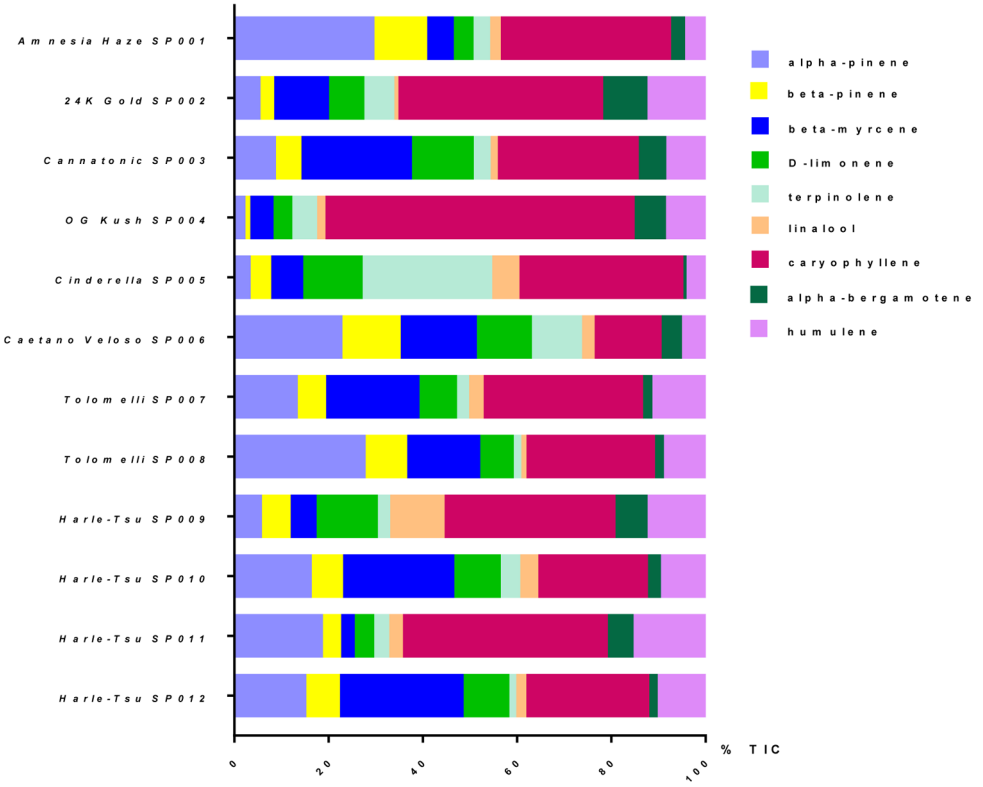
Figure 1 – Chromatographic profile of *Cannabis* variety Tolomelli, SP008.

Table 2 – Terpenes identified in Tolomelly variety, SP008.

Peak[1]	Name	Chemical Structure	Retention Time	Cal RI	Lit RI	Similarity Index
1	α-Pinene		5.642	948	940	97
2	Camphene		6.008	954	953	97
3	β-Pinene		6.567	943	964	97
4	β-Myrcene		6.825	958	955	96
5	D-Limonene		7.467	1018	-	97
8	Terpinolene		8.342	1052	1085	96
9	Linalool		8.483	1082	1080	97
10	Fenchol		8.683	1110	1121	95
16	Santalene		11.682	1412	1420	96
17	Caryophyllene		11.808	1494	1444	95

Peak[1]	Name	Chemical Structure	Retention Time	Cal RI	Lit RI	Similarity Index
18	α-Bergamotene		11.867	1430	1433	97
20	Humulene		12.175	1579	1477	96
23	β-Elemene		12.567	1398	-	91
24	β -Bisabolene		12.667	1500	1506	92
25	α -Gurjunene		13.000	1419	1408	90
26	Valencene		13.183	1474	1491	92
27	Alloaromadendrene		13.283	1386	1453	96
28	Selina-3,7(11)-diene		13.302	1538	1542	96
29	Guaiol		14.023	1612	1605	92
30	Eudesmol		14.400	1656	1655	94

Note: [1]peaks identified in SP008, Figure 1; Cal RI: Calculated Retention Index; Lit RI: Literature Retention Index.



Descriptive statistics for relative percentage TIC

	α-pinene	β-pinene	β-myrcene	D-limonene	terpinolene	linalool	caryophyllene	α-bergamotene	humulene
Mean	13.8	6.3	13.5	8.7	6.0	3.3	34.5	4.2	9.5
Range	2.0 – 29.4	1.0 – 12.4	2.8 – 26.3	4.0 – 13.1	1.5 – 27.5	0.9 – 11.6	14.2 – 65.6	0.7 – 9.4	4.4 – 15.7
SD	9.3	3.3	8.4	3.5	7.2	2.9	12.8	2.6	3.5

SD: standard deviation; TIC: total ion current.

Figure 2 – Distribution of seven terpenes present in *Cannabis* samples.

(indoor and outdoor) by different growers and in different geographical regions. The hour of harvest and dry conditions (time between 7 and 15 days) were also variables and even within this variability nine terpenes were in all samples. The results indicate the potential of the group of nine terpenes composed by α-pinene, β-pinene, β-myrcene, D-limonene, terpinolene, linalool, caryophyllene, α-bergamotene and humulene as complementary markers in the identification of *Cannabis*.

In conclusion, the present study showed the application of a simple and effective method for terpenes analysis in *Cannabis* specimens that serves as a tool for screening procedure in quality control of the raw material and help the taxonomic discrimination. Additionally, the collected information may contribute to research studies about the alleged entourage effect of the different components in the plants.

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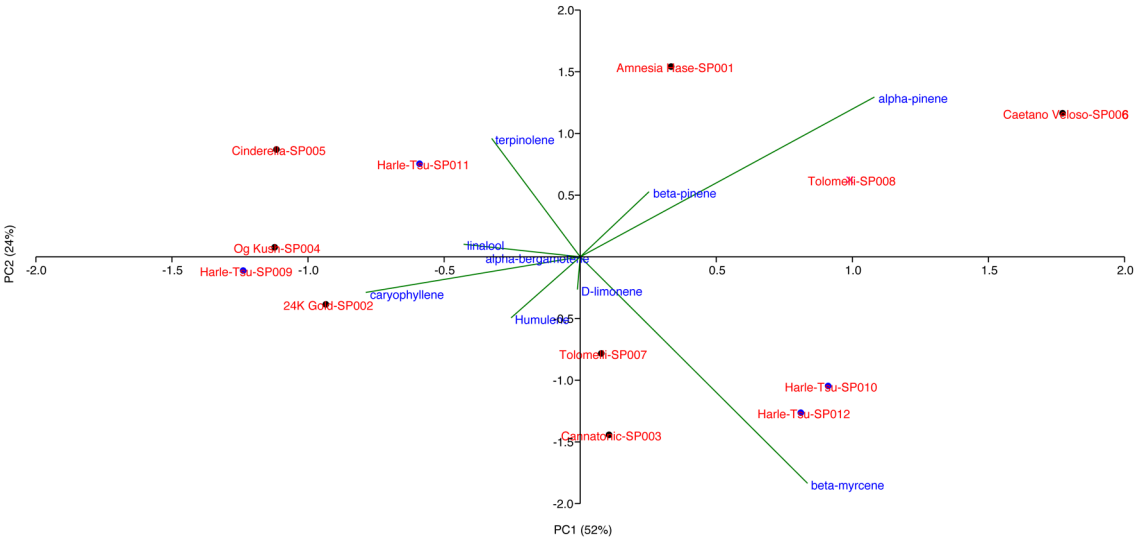

Figure 3 – Principal Component Analysis (PCA) of nine terpenes identified in Cannabis samples cultivated by patients.

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