Article

Not peer-reviewed version

Comparison of Three Gas Chromatographic Methods – Identification of Terpenes and Terpenoids in *Cannabis sativa* L.

Lumír Ondřej Hanuš *

Posted Date: 7 June 2024

doi: 10.20944/preprints202406.0373.v1

Keywords: Gas chromatography; liquid sample; head-space; SPME; terpenes; terpenoids

 doi:10.20944/preprints202406.0373.v1

Article

Comparison of Three Gas Chromatographic Methods – Identification of Terpenes and Terpenoids in *Cannabis sativa* L.

Lumír Ondřej Hanuš

Lumir Lab, Asana Bio Group Ltd., The Hadassah Medical Center, Hebrew University Biotechnology Park, Ein Kerem Campus, Jerusalem 91120, Israel
* Correspondence: hlumir@gmail.com, telephone: +972-2-6439935

Abstract: Terpenes and terpenoids content in cannabis plant was already studied in the past with three used methods. Since these works did not compare the content of these substances under the same conditions, we tried to make this comparison exactly. Three different gas chromatography/mass spectrometry (GS/MS) methods - hexane based liquid extraction (Liq), static head-space extraction (HS), and head-space solid phase microextraction (SPME) – were compared to identify volatile compounds in four different cannabis chemotypes - Green fields, Titan chemotype, Black Domina chemotype, and Neptune chemotype. The main compounds focused on were monoterpenes/monoterpenoids and sesquiterpenes/sesquiterpenoids. Extraction with hexane gave comparable results, however the other two methods allowed for the identification of more substances. For the final evaluation of the comparison of the three methods of analysis, extraction with hexane gives comparable results (which is advantageous for quantitative analysis), although the other two methods allowed for the identification of more substances. This means that the same method should be used everywhere for the quantitative evaluation of constituents in cannabis.

Keywords: gas chromatography; liquid sample; head-space; SPME; terpenes; terpenoids

1. Introduction

Today we know, without any doubt, that the presence of terpenes and terpenoids in the Cannabis sativa L. plant is important, not only from the biogenetical point of view but also from the medical perspective. These bioactive compounds play an important role concerning use of cannabis as a medicament. Unfortunately, up-to-date there is not enough knowledge concerning the importance of these compounds and their ratio with others bioactive compounds, mainly cannabinoids. At present, it is very important to clarify the importance of the compounds quantity, the content compound types and their ratios, and to understand the medicinal power of this plant for the treatment of various diseases.

The biosynthesis of terpenes in cannabis is comprised of two different pathways [1,2] . The first pathway is the plastidial methylerythritol phosphate pathway [3], which starts with the substrates pyruvate and glyceraldehyde-3-phosphate [4,5]. Through several steps geranyl diphosphate is produced, which then interacts with olivetolic acid for cannabigerolic acid origination. Additionally, this pathway is also a precursor for monoterpene origination [6,7]. The second pathway is the cytosolic mevalonate pathway [8] which starts with acetoacetyl-CoA and after several steps forms farnesyl diphosphate followed by sesquiterpenes formation.

Terpenes found in hemp are not unique to this plant, as they are also found in other plants. Many terpenes have medical potential but their bioactivity obviously depends on the cannabis chemotype, due to terpenes and cannabinoids having different quantitative contents and ratios [9–15]. Of course, both the cannabis chemotype used and the patient's genetics play a major role in the treatment. In addition, the amount used in one patient may be too high and in another one insufficient. At present, we need to know much more about the biodynamic effect of terpenes in cannabis in order to be able

 doi:10.20944/preprints202406.0373.v1

to breed cannabis plants that are suitable for therapeutic use. We must understand that the therapeutic effectiveness of terpenes contained in cannabis is different when we use plant material (by smoking, vaporization or in capsules), or as extracts (in oil, capsules, suppositories, creams and the other preparations). We must also understand that many terpenes are not stable compounds. It is possible that some of these phytochemicals are artifacts that formed in the resin on the plant due to various reasons (as described below). For instance, UV sunlight exposure, harvesting, drying, storage and processing of the plant, and even during the analysis. Smoking and vaporization gives rise to other substances, not fully studied, that can also affect patients (or other users) either positively or negatively. So far, there is a discussion on whether terpenes act as such or have a synergistic or entourage effect [16–21].

LaVigne [22] found that the terpenes α-humulene, geraniol, linalool, and β-pinene produced cannabinoid tetrad behaviors in mice [23–26], suggesting cannabimimetic activity. Further, some mice behaviors could be blocked by cannabinoid or adenosine receptor antagonists, suggesting a mixed mechanism of action.

We must take into consideration that upon aging essential oils can undergo oxidation and polymerization which may result in a loss of pharmacological properties. Heat, light and air can lead to its oxidation, polymerization, isomerization, thermal rearrangement, or dehydrogenation [27]. Inflorescence stored using the novel packaging approach is a significant step towards providing patients with cannabis inflorescence of reproducible and reliable terpene content, an important component of inflorescence efficacy [28].

The aim of this study was to compare three different gas chromatography/mass spectrometry methods - hexane based liquid extraction (Liq), static head-space extraction (HS), and head-space solid phase microextraction (SPME), - in order to determine which method best identified volatile compounds in cannabis samples, mainly monoterpenes/monoterpenoids and sesquiterpenes/sesquiterpenoids. We used four chemotypes to also compare possibilities of content compounds identification in different samples.

2. Experimental

2.1. Methods

Standards: Commercially available standards for α-pinene, camphene, β-pinene, myrcene, Δ^3-carene, α-terpinene, *p*-cymene, limonene, 1,8-cineole, α-ocimene, *trans*-β-ocimene, γ-terpinene, terpinolene, linalool, isopulegol, geraniol, β-caryophyllene, α-humulene, *cis*-nerolidol, *trans*-nerolidol, caryophyllene oxide, guaiol, and α-bisabolol were obtained from Restek (Bellefonte, PA, USA).

Plant material: Dry female flowering tops of four different chemotypes (LOH LL1 - Green fields chemotype, LOH LL2 – Titan chemotype, LOH LL3 – Black Domina chemotype, LOH LL4 – Neptune chemotype) used for medical treatment, cultivated in Israel, were used for analysis. These four varieties were selected randomly to compare different chemotypes.

Sample preparation: The ground plant material was extracted with n-hexane (final concentration was 1 mg/ml) with occasional shaking for half an hour. One microgram of the sample thus prepared was injected for Liq analysis. 25 mg of plant material was used for HS and 0.3 mg for SPME analysis.

Instrument: GC/MS [Agilent 7890B GC, Agilent 5977B MSD, PAL 3 (RSI 85)].

Column: Agilent Technologies, Inc., HP-5MS UI, 30 m x 250 μm, film 0.25 μm.

2.2. Experimental Conditions for HS

Incubation time: 6 min; Incubation temperature: 80ºC.

2.3. Experimental Conditions for SPME:

Incubation time: 10 min, incubation temperature: 60ºC, GC cycle time: 5 min, fiber conditioning station temperature: 250 ºC, pre desorption conditioning time: 2 min, sample extraction time: 10 min, sample desorption time: 1 min

2.4. Experimental Condition for All Three Analyses

 doi:10.20944/preprints202406.0373.v1

The column temperature was initially 35 °C for 5 min, followed by temperature ramping from 35-150 °C at 5 °C/min, then to 250 °C at 15 °C/min (inlet: 250 °C; detector: 280 °C; split ratio 5:1;); gas: helium (flow rate: 1 mL/min).

Analytical method validation - selectivity, specificity, accuracy, precision, linearity, range, limit of detection, limit of quantification, ruggedness, and robustness were performed [29]. They are beyond the scope of this manuscript and will be published in another publication.

2.5. Identification

The content compounds were identified by comparison to standards, retention times, retention indices, mass spectra of particular compounds, and the spectral matching of libraries NIST/EPA/NIH Mass Spectral Library 2017, Wiley Registry of Mass Spectral Data 11th Edition, FFNSC3, ©2015, and Adams Essential Oils Library.

3. Results

As we did not have all the main compounds as standards, it was impossible to quantify all these terpenes/terpenoids exactly. We therefore "quantified" volatile compounds by a normalization method. We chose the biggest peak (by space) as 100% and calculated the relative ratios of the other ones (in Tables as. % norm). The ten main peaks in each method are marked in Tables below in bold.

Sample LOH LL1 (Table 1) revealed β-myrcene as the biggest peak in HS and β-caryophyllene in Liq and SPME methods. There are 7 different compounds above 50%; in norm Liq – 7 ones above 50%, in HS – 1 above 50%, and in SPME – 6 above 50%. Between the ten main compounds above 50% were identified β-myrcene - 3 x, β-caryophyllene – 2 x, γ-elemene – 1 x, α-humulene – 2 x, α-bulnesene 2 x, γ-selinene – 2 x, selina-3,7(11)-diene - 2 x. Altogether within the ten main compounds 14 different terpenes/terpenoids were identified. Altogether 51 compounds were identified by HS (97.53% of total volatiles), 46 compounds by SPME (88.36%) and 37 compounds by liquid (72.73%) GC/MS, what was together 67 different volatile compounds. The same 28 compounds were found in all three analyses. Comparison of all three methods of analysis is presented in Chromatogram 1 - Liq (Chromatogram 1a), HS (Chromatogram 1b), and SPME (Chromatogram 1c).

Table 1. GC/MS identification of the dry flowering tops - sample **LOH LL1** chemotype**.**

Peak	RT	% norm (Liq)	% norm (HS)	% norm (SPME)	Compounds	Type	RI
1	6.429		0.02		2,3-butanediol	glycol	788
2	7.789	0.72			2,4-dimethylheptane	hydrocarbon	821
3	9.212	0.20			ethylbenzene	aromatic hydrocarbon	855
4	9.379	0.48			4-methyloctane	hydrocarbon	863
5	9.509	0.75			*p*-xylene	aromatic hydrocarbon	865
6	9.524		0.08		1-hexanol	organic alcohol	868
7	10.718	0.37	0.05		heptanal	alkyl aldehyde	901
8	11.223	0.92	1.29		5,5-dimethyl-1-vinylbicyclo[2.1.1]hexane	bicyclic monoterpene	921
9	11.600		0.04		α-thujene	bicyclic monoterpene	929
10	11.808	4.56	**5.55**	0.54	**α-pinene**	bicyclic monoterpene	937
11	12.329	1.35	1.52	0.26	**camphene**	bicyclic monoterpene	952
12	12.810		0.02		benzaldehyde	aromatic aldehyde	962
13	13.372	8.38	**10.07**	1.50	**β-pinene**	bicyclic monoterpene	979

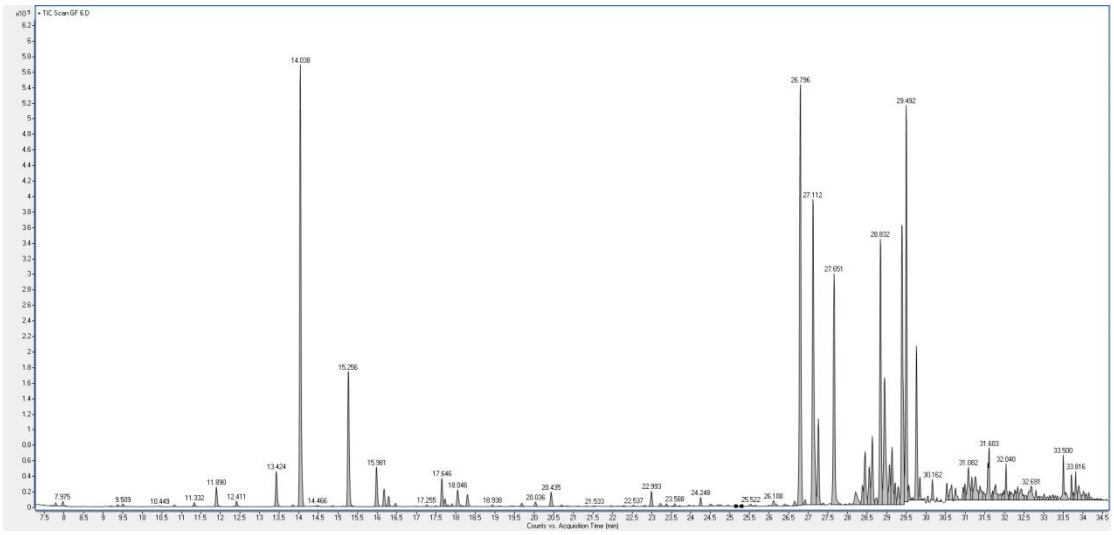
 doi:10.20944/preprints202406.0373.v1

14	13.893		0.03		6-methyl-5-hepten-2-one	unsaturated methylated ketone	986
15	14.053	**95.78**	**100.00**	**86.64**	**β-myrcene**	acyclic monoterpene	991
16	14.887			0.23	**α-terpinene**	monocyclic monoterpene	1017
17	15.111		0.02	0.08	***p*-cymene**	monocyclic monoterpene	1025
18	15.247	**32.07**	**30.90**	**30.66**	**limonene**	monocyclic monoterpene	1030
19	15.328		0.13		**1,8-cineole**	bicyclic monoterpenoid	1032
20	15.624		0.06		*cis*-β-ocimene	acyclic monoterpene	1038
21	15.969		0.01	0.19	***trans*-β-ocimene**	acyclic monoterpene	1049
22	16.273		0.06	0.15	**γ-terpinene**	monocyclic monoterpene	1060
23	16.554		0.04		sabinene hydrate	bicyclic monoterpenoid	1068
24	17.243		0.34	1.13	**terpinolene**	monocyclic monoterpene	1088
25	17.636	6.38	2.90	2.86	**linalool**	acyclic monoterpenoid	1099
26	18.037	4.64	1.29	3.46	fenchyl alcohol	bicyclic monoterpenoid	1113
27	18.286	3.07	0.71		*cis*-pinene hydrate	bicyclic monoterpenoid	1121
28	18.590			0.25	*neo-allo*-ocimene	acyclic monoterpene	1131
29	19.079		0.03		ipsdienol	acyclic monoterpenoid	1147
30	19.672	0.96	0.15	0.52	borneol	bicyclic monoterpenoid	1166
31	20.025	1.12	0.20	0.70	terpinen-4-ol	monocyclic monoterpenoid	1177
32	20.426	3.40	0.39	0.91	α-terpineol	monocyclic monoterpenoid	1189
33	20.706			0.10	dodecane	alkane hydrocarbon	1200
34	24.931			0.06	α-cubebene	tricyclic sesquiterpene	1351
35	25.516	0.56	0.09	1.18	ylangene	tricyclic sesquiterpene	1372
36	25.637	0.23	0.07	0.63	α-copaene	tricyclic sesquiterpene	1376
37	25.813			0.18	β-patchoulene	tricyclic sesquiterpene	1381
38	25.997		0.03	0.31	7-*epi*-sesquithujene	bicyclic sesquiterpene	1391
39	26.206			0.31	tetradecane	alkane hydrocarbon	1400
40	26.462		0.03	1.56	*cis*-β-caryophyllene	bicyclic sesquiterpene	1406
41	26.639	1.11	0.19	3.33	*cis*-α-bergamotene	bicyclic sesquiterpene	1415
42	26.791	**100.00**	**12.73**	**100.00**	**β-caryophyllene**	bicyclic sesquiterpene	1419
43	26.879		0.02		γ-maaliene	tricyclic sesquiterpene	1430
44	27.024		0.02		β-copaene	tricyclic sesquiterpene	1433
45	27.144	**71.31**		14.69	γ-elemene	monocyclic sesquiterpene	1434
46	27.160	5.60	1.35	15.36	α-bergamotene	bicyclic sesquiterpene	1436
47	27.248	**19.06**	2.44	**25.89**	α-guaiene	bicyclic sesquiterpene	1439
48	27.376	0.43	0.06	1.39	guaia-6,9-diene	bicyclic sesquiterpene	1443
49	27.585			2.32	humulen-(v1)	bicyclic sesquiterpene	1455
50	27.649	**55.13**	**5.56**	**61.27**	**α-humulene**	monocyclic sesquiterpene	1454

 doi:10.20944/preprints202406.0373.v1

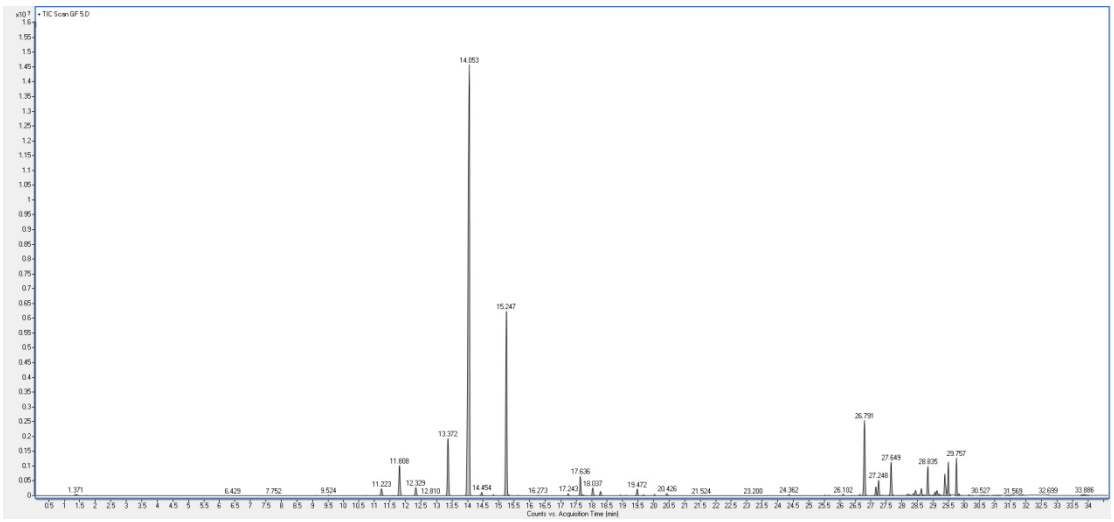
51	28.042	0.27	0.02	0.86	4,5-di-epi-aristolochene	bicyclic sesquiterpene	1467
52	28.194	6.30	0.39	6.68	γ-muurolene	bicyclic sesquiterpene	1477
53	28.282		0.07		α-amorphene	bicyclic sesquiterpene	1485
54	28.370	4.35	0.36	5.68	4a,8-Dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene	bicyclic sesquiterpene	1492
55	28.434	13.06	0.92	**16.48**	β-selinene	bicyclic sesquiterpene	1486
56	28.555			3.73	δ-selinene	bicyclic sesquiterpene	1488
57	28.627	16.16	1.21	**23.47**	α-selinene	bicyclic sesquiterpene	1494
58	28.835	**56.69**	**4.39**	**56.51**	α-bulnesene	bicyclic sesquiterpene	1505
59	28.988		0.08	1.86	γ-cadinene	bicyclic sesquiterpene	1513
60	29.124	13.65	0.87	16.32	δ-amorphene	bicyclic sesquiterpene	1519
61	29.380	**69.01**	**3.66**	**67.76**	γ-selinene	bicyclic sesquiterpene	1544
62	29.493	**73.42**	**4.35**	**89.15**	selina-3,7(11)-diene	bicyclic sesquiterpene	1542
63	29.757	**31.07**	**4.40**	0.37	germacrene B	monocyclic sesquiterpene	1557
64	30.158	3.53	0.08		**caryophyllene oxide**	bicyclic sesquiterpenoid	1581
65	31.312			0.57	cadalene	bicyclic aromatic hydrocarbon	1674
66	31.575	4.56			juniper camphor	bicyclic sesquiterpenoid	1691
67	32.378			0.17	guaiazulene	bicyclic sesquiterpene	1775

Chromatogram 1a. Hexane based liquid extraction method (Liq) - sample **LOH LL1** chemotype. Counts vs. Acquisition Time (min)

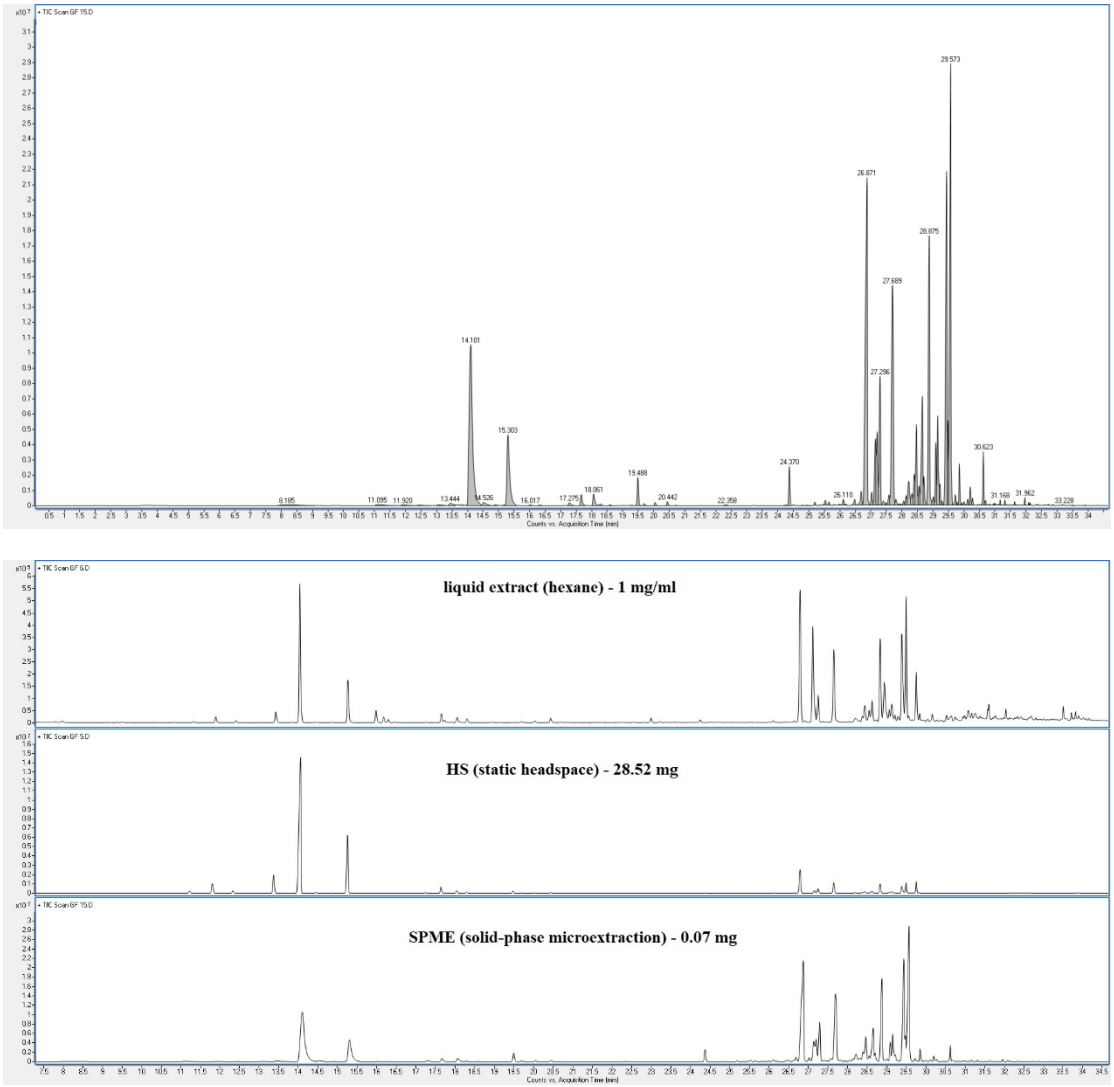


Chromatogram 1b. Static head-space extraction method (HS) - sample **LOH LL1** chemotype. Counts vs. Acquisition Time (min)

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 7 June 2024 doi:10.20944/preprints202406.0373.v1



Chromatogram 1c. Head-space solid phase microextraction method (SPME) - sample **LOH LL1** chemotype. Counts vs. Acquisition Time (min)



Chromatogram 1. Comparison of all three methods of analysis - sample **LOH LL1** chemotype. Counts vs. Acquisition Time (min).

Results of analysis of the sample LOH LL2 are presented in Table 2. Limonene was the biggest peak in HS and β-caryophyllene in Liq and SPME methods. Three different compounds were above

 doi:10.20944/preprints202406.0373.v1

50%; in norm Liq – 3 above 50%, in HS – 1 above 50%, and in SPME – 2 above 50%. Of the ten main compounds identified above 50% were limonene 1 x, β-caryophyllene – 2 x, and α-humulene – 1 x. Altogether 17 different terpenes/terpenoids were found between the ten main compounds. The same 24 compounds were found in all three analyses. Altogether 74 compounds were identified by HS (97.87% of total volatiles), 57 compounds by SPME (90.80%) and 38 compounds by liquid (80.02%) GC/MS, what was together 94 different volatile compounds. Comparison of all three methods of analysis is presented in Chromatogram 2.

Table 2. GC/MS identification of the dry flowering tops - sample **LOH LL2** chemotype.

Peak	RT	% norm (Liq)	% norm (HS)	% norm (SPME)	Compound	Type	RI
1	2.966		0.12		2-methylbutanal	saturated fatty aldehyde	662
2	4.578		0.21		2-methyl-1-butanol	alcohol	739
3	6.373		0.67		DL-2,3-butanediol	glycol	773
4	6.510		0.11		2,3-butanediol	glycol	788
5	7.799	0.12			2,4-dimethyl-heptane	hydrocarbon	821
6	9.508		0.53		1-hexanol	alcohol	868
7	10.718		0.16		heptanal	saturated fatty aldehyde	901
8	11.231		0.32		5,5-dimethyl-1-vinylbicyclo[2.1.1]hexane	bicyclic monoterpene	921
9	11.608		0.09		α-thujene	bicyclic monoterpene	929
10	11.808	4.55	**15.82**	0.93	**α-pinene**	bicyclic monoterpene	937
11	12.33	1.39	**4.88**	0.98	**camphene**	bicyclic monoterpene	953
12	12.811		0.05		benzaldehyde	aromatic aldehyde	962
13	13.372	7.74	**25.48**	1.24	**β-pinene**	bicyclic monoterpene	979
14	13.901		0.75		2,2,4,6,6-pentamethylheptane	hydrocarbon	991
15	14.013	**10.24**	**37.32**	**8.73**	**β-myrcene**	acyclic monoterpene	991
16	14.358		0.12		ethyl hexanoate	fatty acid ester	1000
17	14.839			0.52		monocyclic	
			0.16		**α-terpinene**	monoterpene	1017
18	14.983					monocyclic	
			0.05		*p*-menth-1-ene	monoterpene	1025
19	15.111			0.74		monocyclic	
			0.07		***p*-cymene**	monoterpene	1025
20	15.272	**36.55**		**23.48**		monocyclic	
			100.00		**limonene**	monoterpene	1030
21	15.336		0.31		**1,8-cineole**	bicyclic monoterpenoid	1032
22	15.624		0.20	0.13	*cis*-β-ocimene	acyclic monoterpene	1038
23	16.017			0.65	***trans*-β-ocimene**	acyclic monoterpene	1049
24	16.282			0.29		monocyclic	
			0.11		**γ-terpinene**	monoterpene	1060
25	16.546		0.21		cis-sabinene hydrate	bicyclic monoterpenoid	1070

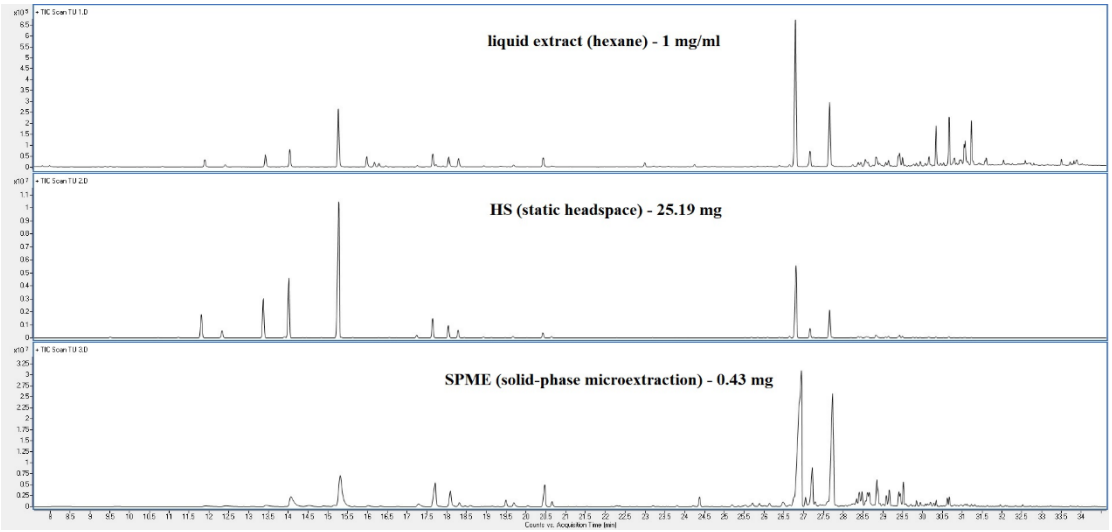
 doi:10.20944/preprints202406.0373.v1

26	16.739					monocyclic	
			0.13		linalool oxide	monoterpenoid	1086
27	17.283			1.69		monocyclic	
					terpinolene	monoterpene	1088
28	17.644	**7.96**	**11.36**	**11.42**	**linalool**	acyclic monoterpenoid	1099
29	17.730	1.53			undecane	hydrocarbon	1100
30	17.789		0.06		nonanal	saturated fatty aldehyde	1104
31	18.037	7.21	**7.27**	**6.74**	fenchol	bicyclic monoterpenoid	1113
32	18.286	5.65	4.61	1.64	*cis*-pinene hydrate	bicyclic monoterpenoid	1121
33	18.438		0.19	0.25	methyl octanoate	fatty acid ester	1126
34	18.590			0.49	*neo-allo*-ocimene	acyclic monoterpene	1131
35	18.919	0.67	0.53		*trans*-pinene hydrate	bicyclic monoterpenoid	1140
36	19.015		0.07		camphor	bicyclic monoterpenoid	1145
37	19.119		0.22	0.20	camphene hydrate	bicyclic monoterpenoid	1148
38	19.408		0.03		isoborneol	bicyclic monoterpenoid	1157
39	19.665	1.42	0.94	1.69	borneol	bicyclic monoterpenoid	1166
40	20.025			0.32		monocyclic	
			0.11		terpinen-4-ol	monoterpenoid	1177
41	20.418	5.93		**7.99**		monocyclic	
			3.01		α-terpineol	monoterpenoid	1189
42	20.635	0.89	0.79	1.63	ethyl octanoate	fatty acid ester	1196
43	23.192		0.12	0.32	bornyl acetate	bicyclic monoterpenoid	1286
44	23.801		0.07	0.33	(*E*)-4-decenoic acid methyl ester	fatty acid ester	1299
45	24.202		0.04	0.32	methyl decanoate	fatty acid ester	1325
46	24.939			0.10	α-cubebene	tricyclic sesquiterpene	1351
47	25.525		0.15	0.79	ylangene	tricyclic sesquiterpene	1372
48	25.685		0.33	1.60	ethyl *trans*-4-decenoate	fatty acid ester	1375
49	25.837		0.28	1.20	hexyl hexanoate	fatty acid ester	1384
50	25.990		0.17	0.43	7-*epi*-sesquithujene	bicyclic sesquiterpene	1391
51	26.086		0.30	1.27	ethyl decanoate	fatty acid ester	1396
52	26.246			0.07	tetradecane	hydrocarbon	1400
53	26.302			0.15	cyperene	tricyclic sesquiterpene	1399
54	26.390	0.79	0.26		sesquithujene	bicyclic sesquiterpene	1402
55	26.463		0.17	2.37	*cis*-caryophyllene	bicyclic sesquiterpene	1406
56	26.647	1.33	0.97		*cis*-α-bergamotene	bicyclic sesquiterpene	1415
57	26.807	**100.00**	**48.85**	**100.00**	**β-caryophyllene**	bicyclic sesquiterpene	1419
58	27.047			2.19	10,10-dimethyl-2,6-dimethylenebicyclo[7.2.0]undecane	bicyclic sesquiterpene	1440
59	27.16	**10.57**	**5.28**	**14.84**	α-bergamotene	bicyclic sesquiterpene	1435
60	27.248	0.62	0.26	1.15	α-guaiene	bicyclic sesquiterpene	1439

 doi:10.20944/preprints202406.0373.v1

61	27.376		0.18		guaia-6,9-diene	bicyclic sesquiterpene	1444
62	27.465		0.06		*epi*-β-santalene	bicyclic sesquiterpene	1448
63	27.480			0.30	α-himachalene	bicyclic sesquiterpene	
64	27.649	**42.09**		**55.63**		monocyclic	
			17.38		**α-humulene**	sesquiterpene	1454
65	27.809			0.24	β-santalene	bicyclic sesquiterpene	1462
66	28.338			2.08	α-curcumene	aromatic sesquiterpene	1483
67	28.370	2.17	0.85		selina-4,11-diene	bicyclic sesquiterpene	1474
68	28.402			4.05	4a,8-dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene	bicyclic sesquiterpene	1492
69	28.443	2.29	0.67	4.04	β-selinene	bicyclic sesquiterpene	1486
70	28.587		0.84	4.19	valencene	bicyclic sesquiterpene	1492
71	28.627	1.93	0.67	3.90	α-selinene	bicyclic sesquiterpene	1494
72	28.747		0.09	0.36	β-dihydroagarofuran	tricyclic sesquiterpenoid	1496
73	28.819	7.14	2.28	**9.18**	α-farnesene	acyclic sesquiterpene	1508
74	28.931			0.62		monocyclic	
					β-curcumene	sesquiterpene	1514
75	28.972		0.11		sesquicineole	bicyclic sesquiterpenoid	1516
76	29.140	3.00		4.59		monocyclic	
			0.88		β-sesquiphellandrene	sesquiterpene	1524
77	29.389	4.11	0.36	3.48	γ-selinene	bicyclic sesquiterpene	1538
78	29.493	3.88	0.83	**5.12**	selina-3,7(11)-diene	bicyclic sesquiterpene	1542
79	29.757					monocyclic	
			0.29		germacrene B	sesquiterpene	1557
80	29.781	0.94			nerolidol	acyclic sesquiterpenoid	1544
81	30.158	4.86	0.51		**caryophyllene oxide**	bicyclic sesquiterpenoid	1581
82	30.334	**16.49**	0.46	1.11	**guaiol**	bicyclic sesquiterpenoid	1596
83	30.455	0.84	0.02		5-*epi*-7-*epi*-α-eudesmol	bicyclic sesquiterpenoid	1616
84	30.527		0.06		humulene epoxide II	bicyclic sesquiterpenoid	1606
85	30.663	**22.81**	0.55	1.91	10-*epi*-γ-eudesmol	bicyclic sesquiterpenoid	1619
86	30.799	5.38	0.04		γ-eudesmol	bicyclic sesquiterpenoid	1631
87	30.859	1.07			agarospirol	bicyclic sesquiterpenoid	1645
88	31.040	7.59	0.07		β-eudesmol	bicyclic sesquiterpenoid	1649
89	31.072	**13.73**	0.11		α-eudesmol	bicyclic sesquiterpenoid	1653
90	31.224	**19.51**	0.09	0.35	bulnesol	bicyclic sesquiterpenoid	1667
91				0.34		bicyclic aromatic	
	31.320				cadalene	hydrocarbon	1674
92	31.575	1.62			juniper camphor	bicyclic sesquiterpenoid	1691
93	32.523			0.31	α-phellandrene dimer	tricyclic terpene	1801
94	33.733			0.13	hexadecanoic acid	saturated fatty acid	1968

 doi:10.20944/preprints202406.0373.v1



Chromatogram 2. Comparison of all three methods of analysis - sample **LOH LL2** chemotype. Counts vs. Acquisition Time (min).

In Table 3 limonene was the biggest peak in HS and SPME and selina-3,7(11)-diene in Liq. Five different compounds were above 50%; in norm Liq – 4 above 50%, in HS – 2 above 50%, and in SPME – 5 above 50%. Between the ten main compounds identified using all three methods was above 50% β-myrcene – 2 x, limonene – 3 x, β-caryophyllene – 2 x, γ-selinene – 2 x, selina-3,7(11)-diene – 2 x. Altogether there were 20 different terpenes/terpenoids between the ten main compounds. The same 21 compounds were found in all three types of analysis. Altogether 55 compounds were identified by HS (96.45%), 49 compounds by SPME (85.43%) and 45 compounds by Liq (86.85%) GC/MS, what was together 84 different volatile compounds. Comparison of all three methods of analysis is presented in Chromatogram 3.

Table 3. GC/MS identification of the dry flowering tops - sample **LOH LL3** chemotype.

Peak	RT	% norm (Liq)	% norm HS	% norm (SPME)	Compound	Type	RI
1	8.987		0.10		3-hexen-1-ol		856
2	9.398	0.51			4-methyl-octane	hydrocarbon	863
3	9.528	0.74			*p*-xylene	aromatic hydrocarbon	865
4	9.508	1.02	1.70	1.58	1-hexanol	organic alcohol	868
5	11.239		0.21		5,5-dimethyl-1-vinylbicyclo[2.1.1]hexane	bicyclic monoterpene	921
6	11.608		0.50	0.45	α-thujene	bicyclic monoterpene	929
7	11.824	11.86	**24.64**	4.11	**α-pinene**	bicyclic monoterpene	937
8	12.345	3.43	**7.13**	0.93	**camphene**	bicyclic monoterpene	952
9	13.388	17.21	**33.51**	4.92	**β-pinene**	bicyclic monoterpene	979
10	14.045	**41.18**	**76.91**	**70.38**	**β-myrcene**	acyclic monoterpene	991
11	14.406		0.11		α-phellandrene	monocyclic monoterpene	1005
12	14.646			**29.77**	**Δ³-carene**	bicyclic monoterpene	1011
13	14.879			2.48	**α-terpinene**	monocyclic monoterpene	1017
14	14.999		0.10		*p*-menth-1-ene	monocyclic monoterpene	1025

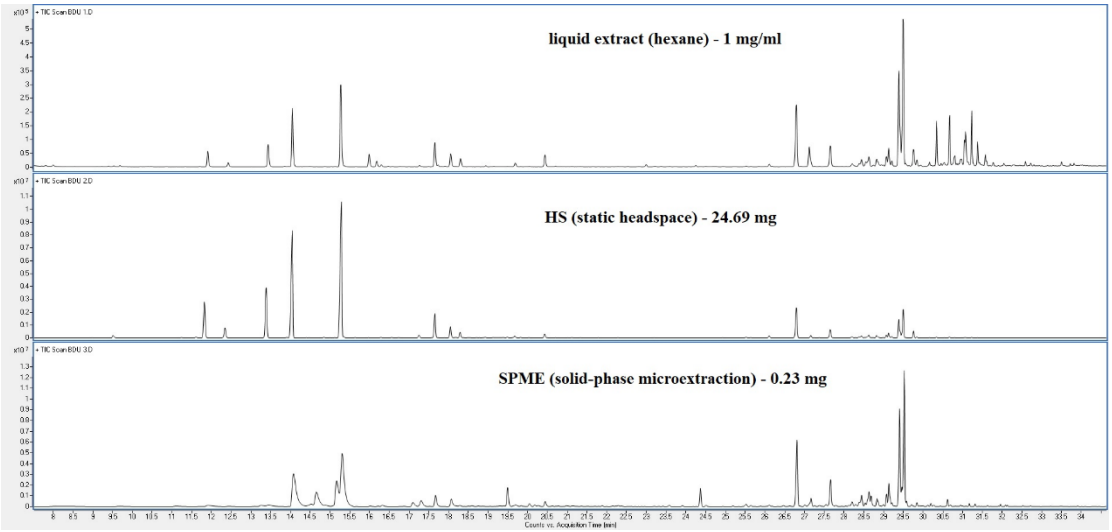
 doi:10.20944/preprints202406.0373.v1

15	15.119		0.13	**38.91**	***p*-cymene**	monocyclic monoterpene	1025
16	15.288	**63.52**	**100.00**	**100.00**	**limonene**	monocyclic monoterpene	1030
17	15.344		0.75		**1,8-cineole**	bicyclic monoterpenoid	1032
18	15.632		0.20		*cis*-β-ocimene	acyclic monoterpene	1038
19	15.977		0.10	0.79	***trans*-β-ocimene**	acyclic monoterpene	1049
20	16.290		0.25	1.92	**γ-terpinene**	monocyclic monoterpene	1060
21	16.554		0.21		*cis*-sabinene hydrate	bicyclic monoterpenoid	1070
22	16.322			0.42	*p*-cresol	phenol derivative	1077
23	16.875			5.67	*m*-cymenene	aromatic compound	1082
24	17.252		1.68		**terpinolene**	monocyclic monoterpene	1088
25	17.308			8.92	*p*-cymenene	aromatic compound	1090
26	17.652	**18.06**	**14.53**	11.77	**linalool**	acyclic monoterpenoid	1099
27	17.730	1.46			undecane	hydrocarbon	1100
28	18.045	11.22	**7.01**	8.98	fenchol	bicyclic monoterpenoid	1113
29	18.286	6.74	3.40		*trans*-pinene hydrate	bicyclic monoterpenoid	1132
30	19.127	0.40	0.22		camphene hydrate	bicyclic monoterpenoid	1148
31	18.871			0.34	5-methyl-undecane	hydrocarbon	1156
32	19.296		0.17		2,3-dimethyldecane	hydrocarbon	1157
33	19.673	3.14	1.34	2.52	borneol	bicyclic monoterpenoid	1166
34	19.817		0.25	0.70	3-methyl-undecane	hydrocarbon	1170
35	20.025			2.48		monocyclic	
			0.15		terpinen-4-ol	monoterpenoid	1177
36	20.178			1.34		monocyclic	
					p-cymene-8-ol	monoterpenoid	1183
37	20.426	8.93		4.41		monocyclic	
			2.37		α-terpineol	monoterpenoid	1189
38	20.627			0.34	myrtenal	bicyclic monoterpenoid	1193
39	20.705	0.56			dodecane	hydrocarbon	1200
40	23.192		0.10		bornyl acetate	bicyclic monoterpenoid	1286
41	23.585			1.08	carvacrol	monoterpenoid phenol	1299
42	24.931		0.10		α-cubebene	tricyclic sesquiterpene	1351
43	25.525	0.57	0.36	2.34	ylangene	tricyclic sesquiterpene	1372
44	25.637		0.15	0.71	copaene	tricyclic sesquiterpene	1376
45	25.845		0.08		hexyl hexanoate	fatty acid ester	1384
46	25.893			0.22	α-bourbonene	tricyclic sesquiterpene	1384
47	26.182	0.42			tetradecane	hydrocarbon	1400
48	26.471			0.69	*cis*-β-caryophyllene	bicyclic sesquiterpene	1406
49	26.791	**50.61**	**19.34**	**54.32**	**β-caryophyllene**	bicyclic sesquiterpene	1419
50	27.121	14.06		1.55	γ-elemene	monocyclic sesquiterpene	1434
51	27.160	3.24	1.49	6.27	α-bergamotene	bicyclic sesquiterpene	1435

 doi:10.20944/preprints202406.0373.v1

52	27.248		0.10	0.49	α-guaiene	bicyclic sesquiterpene	1439
53	27.376		0.13	0.88	guaia-6,9-diene	bicyclic sesquiterpene	1444
54	27.545			1.70	humulen-(v1)	bicyclic sesquiterpene	1455
55	27.649	17.43	5.37	**22.06**	**α-humulene**	monocyclic sesquiterpene	1454
56	28.202	2.60	0.79	4.01	γ-muurolene	bicyclic sesquiterpene	1477
57	28.290		0.17	1.77	α-amorphene	bicyclic sesquiterpene	1482
58	28.386			2.92	4a,8-dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene	bicyclic sesquiterpene	1492
59	28.378	1.49	0.52		selina-4,11-diene	bicyclic sesquiterpene	1474
60	28.443	5.84	1.34	9.13	β-selinene	bicyclic sesquiterpene	1486
61	28.531		0.22		δ-selinene	bicyclic sesquiterpene	1495
62	28.627	8.87	2.26	**13.55**	α-selinene	bicyclic sesquiterpene	1494
63	28.749	0.87			β-dihydroagarofuran	tricyclic sesquiterpenoid	1496
64	28.819	7.49	1.74	6.98	α-farnesene	acyclic sesquiterpene	1508
65	28.996	0.73	0.19	1.31	γ-cadinene	bicyclic sesquiterpene	1513
66	29.132			**18.11**	δ-amorphene	bicyclic sesquiterpene	1497
67	29.388	**73.83**	**11.61**	**63.98**	γ-selinene	bicyclic sesquiterpene	1544
68	29.469			9.56	α-bisabolene	monocyclic sesquiterpene	1540
69	29.501	**100.00**	**15.20**	**84.02**	selina-3,7(11)-diene	bicyclic sesquiterpene	1542
70	29.757	12.81	3.04		germacrene B	monocyclic sesquiterpene	1557
71	30.158	2.54	0.12		**caryophyllene oxide**	bicyclic sesquiterpenoid	1581
72	30.334	**22.79**	0.36		**guaiol**	bicyclic sesquiterpenoid	1596
73	30.450	1.30			5-*epi*-7-*epi*-α-eudesmol	bicyclic sesquiterpenoid	1616
74	30.663	**27.80**	0.38		10-*epi*-γ-eudesmol	bicyclic sesquiterpenoid	1619
75	30.799	9.26	0.06		γ-eudesmol	bicyclic sesquiterpenoid	1631
76	30.859	1.46			agarospirol	bicyclic sesquiterpenoid	1645
77	31.045	10.68	0.10		β-eudesmol	bicyclic sesquiterpenoid	1649
78	31.072	**23.23**	0.14		α-eudesmol	bicyclic sesquiterpenoid	1653
79	31.224	**26.57**	0.17		bulnesol	bicyclic sesquiterpenoid	1667
80	31.312			1.17	cadalene	bicyclic aromatic hydrocarbon	1674
81	31.377	14.80	0.08		**α-bisabolol**	monocyclic sesquiterpenoid	1684
82	31.575	7.52			juniper camphor	bicyclic sesquiterpenoid	1692
83	32.523			0.36	α-phellandrene dimer	tricyclic terpene	1801
84	32.719	1.67			selina-4,7-diol	bicyclic sesquiterpenoid	1826

 doi:10.20944/preprints202406.0373.v1



Chromatogram 3. Comparison of all three methods of analysis - sample **LOH LL3** chemotype. Counts vs. Acquisition Time (min).

Table 4 pointed out limonene as the biggest peak in HS and β-caryophyllene in Liq and SPME. Four different compounds were above 50%; in norm Liq – 1 above 50%, in HS – 3 above 50%, and in SPME – 2 above 50%. Between the ten main compounds gathered from all three methods were above 50% β-myrcene – 1 x, limonene – 1 x, β-caryophyllene – 3 x, and α-humulene – 1 x. Altogether 17 different terpenes/terpenoids were identified between the ten main compounds were. The same 21 compounds were found in all three types analysis. Altogether 57 compounds were identified by HS (97.17%), 47 compounds by SPME (88.85%), and 34 compounds by Liq (88.10%) GC/MS, what was together 77 different volatile compounds. Comparison of all three methods of analysis is presented in Chromatogram 4.

Table 4. GC/MS identification of the dry flowering tops - sample **LOH LL4** chemotype.

Peak	RT	% norm (Liq)	% norm (HS)	% norm (SPME)	Compound	Type	RI
1	9.398	0.36			4-methyl-octane	hydrocarbon	863
2	9.515		0.98		1-hexanol	organic alcohol	868
3		0.51			o-xylene	aromatic hydrocarbon	887
4	10.341		0.10		2-heptanone	ketone	891
5	10.734		0.20		heptanal	alkyl aldehyde	901
6	11.239		1.29		5,5-Dimethyl-1-vinylbicyclo[2.1.1]hexane	bicyclic monoterpene	921
7	11.600		1.58	0.52	3-methyl-2-butenoic acid ethyl ester	fatty acid ester	924
8	11.824	3.89	**19.50**	0.81	**α-pinene**	bicyclic monoterpene	937
9	12.345	1.19	5.88	0.99	**camphene**	bicyclic monoterpene	952
10	12.818		0.10		benzaldehyde	aromatic aldehyde	962
11	13.379	5.53	**26.49**		**β-pinene**	bicyclic monoterpene	979
12	13.941			0.23	6-methyl-5-heptene-2-one	unsaturated methylated ketone	986

 doi:10.20944/preprints202406.0373.v1

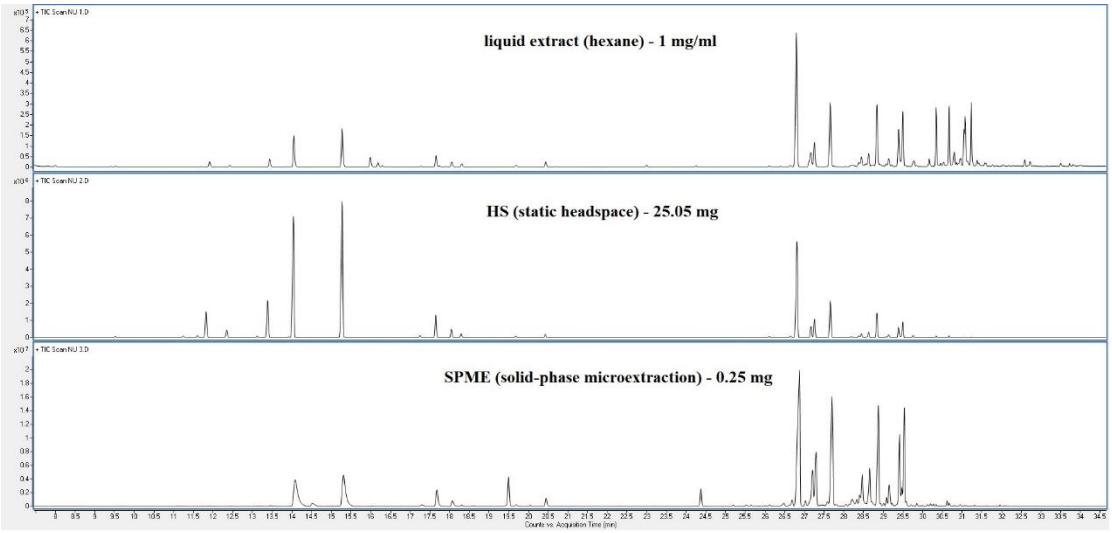
13	13.917				2,2,4,6,6-pentamethylheptane	hydrocarbon	991
			0.73				
14	14.037	20.17	**87.07**	**32.79**	**β-myrcene**	acyclic monoterpene	991
15	14.398					monocyclic	
			0.10		*α*-phellandrene	monoterpene	1005
16	14.846			0.29		monocyclic	
			0.22		**α-terpinene**	monoterpene	1017
17	14.999					monocyclic	
			0.07		*p*-menth-1-ene	monoterpene	1025
18	15.039			0.39	isomyrcenol	acyclic monoterpenoid	1022
19	15.119			0.32		monocyclic	
			0.16		***p*-cymene**	monoterpene	1025
20	15.271	**26.80**		**32.54**		monocyclic	
			100.00		**limonene**	monoterpene	1030
21	15.343		0.53		**1,8-cineole**	bicyclic monoterpenoid	1032
22	15.632		0.22		*cis*-β-ocimene	acyclic monoterpene	1038
23	16.025			0.58	**trans-β-ocimene**	acyclic monoterpene	1049
24	16.289			0.26		monocyclic	
			0.22		**γ-terpinene**	monoterpene	1060
25	16.562		0.16		*cis*-sabinene hydrate	bicyclic monoterpenoid	1070
26	17.291			1.41		monocyclic	
					terpinolene	monoterpene	1088
27	17.644	7.85	**14.47**	10.37	**linalool**	acyclic monoterpenoid	1099
28	17.813			0.19	nonanal	aldehyde	1104
29	18.045	3.93	5.52	3.88	fenchol	bicyclic monoterpenoid	1113
30	18.294	2.38	2.79	0.82	*trans*-pinene hydrate	bicyclic monoterpenoid	1140
31	18.598			0.53	*allo*-ocimene	acyclic monoterpene	1144
32	18.927		0.26		*cis*-pinene hydrate	bicyclic monoterpenoid	1121
33	19.135		0.22		camphene hydrate	bicyclic monoterpenoid	1148
34	19.672	1.34	1.07	1.08	borneol	bicyclic monoterpenoid	1166
35	20.033			0.56		monocyclic	
			0.10		terpinen-4-ol	monoterpenoid	1177
36	20.426	3.63		4.27		monocyclic	
			2.13		*α*-terpineol	monoterpenoid	1189
37	21.147			0.21	2,4-dimethyl-benzaldehyde	aromatic aldehyde	1181
38	24.931		0.10		*α*-cubebene	tricyclic sesquiterpene	1351
39	15.196			0.54	clovene	tricyclic sesquiterpene	1440
40	25.524		0.17	0.81	ylangene	tricyclic sesquiterpene	1372
41	25.645		0.18	0.45	copaene	tricyclic sesquiterpene	1376

 doi:10.20944/preprints202406.0373.v1

42	25.989		0.22	0.36	7-*epi*-sesquithujene	bicyclic sesquiterpene	1391
43	26.390		0.35		sesquithujene	bicyclic sesquiterpene	1402
44	26.462		0.25	2.04	*cis*-caryophyllene	bicyclic sesquiterpene	1406
45	26.647	1.01	1.05		*cis*-α-bergamotene	bicyclic sesquiterpene	1415
46	26.807	**100.00**	**68.80**	**100.00**	**β-caryophyllene**	bicyclic sesquiterpene	1419
47	27.023		0.08		β-copaene	tricyclic sesquiterpene	1432
48	27.168	13.59	7.26	**20.70**	*trans*-α-bergamotene	bicyclic sesquiterpene	1435
49	27.256	17.05	**12.19**	**25.39**	α-guaiene	bicyclic sesquiterpene	1439
50	27.464		0.08		*epi*-β-santalene	bicyclic sesquiterpene	1448
51	27.585			2.17	humulene-(v1)	bicyclic sesquiterpene	1455
52	27.657	**45.78**		**63.07**		monocyclic	
			24.62		**α-humulene**	sesquiterpene	1454
53	28.042		0.11		aristolochene	bicyclic sesquiterpene	1476
54	28.050			0.62	drima-7,9-diene	bicyclic sesquiterpene	1461
55	28.330			3.10		monocyclic	
					α-curcumene	sesquiterpene	1483
56	28.378		1.15	4.70	4a,8-dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene	bicyclic sesquiterpene	1492
57	28.442	7.71	2.95	16.02	β-selinene	bicyclic sesquiterpene	1486
58	28.627	9.44	3.48	**20.62**	α-selinene	bicyclic sesquiterpene	1494
59	28.835	**42.10**	**14.65**	**45.64**	α-bulnesene	bicyclic sesquiterpene	1505
60	29.012			1.09	γ-cadinene	bicyclic sesquiterpene	1513
61	29.380	**25.36**	6.27	**27.14**	γ-selinene	bicyclic sesquiterpene	1538
62	29.468			5.77		monocyclic	
					α-bisabolene	sesquiterpene	1540
63	29.493	**33.77**	**8.89**	**37.76**	selina-3,7(11)-diene	bicyclic sesquiterpene	1542
64	29.757	5.04				monocyclic	
			1.32		germacrene B	sesquiterpene	1557
65	30.158	4.70	0.31		**caryophyllene oxide**	bicyclic sesquiterpenoid	1581
66	30.342	**28.03**	0.82	0.41	**guaiol**	bicyclic sesquiterpenoid	1596
67	30.438			0.09	β-atlantol	monocyclic	1607
						sesquiterpenoid	
68	30.454	2.11	0.04		5-*epi*-7-*epi*-α-eudesmol	bicyclic sesquiterpenoid	1616
69	30.663	**30.65**	0.83	1.04	10-*epi*-γ-eudesmol	bicyclic sesquiterpenoid	1619
70	30.799	11.05	0.22		γ-eudesmol	bicyclic sesquiterpenoid	1631
71	30.859	2.03			agarospirol	bicyclic sesquiterpenoid	1645
72	31.040	15.65	0.24		β-eudesmol	bicyclic sesquiterpenoid	1649
73	31.072	**29.06**	0.45		α-eudesmol	bicyclic sesquiterpenoid	1653
74	31.232	**29.44**	0.42	0.14	bulnesol	bicyclic sesquiterpenoid	1667

 doi:10.20944/preprints202406.0373.v1

				0.30	cadalene	bicyclic aromatic hydrocarbon	1674
75	31.312						
76	31.380	3.67			**α-bisabolol**	monocyclic sesquiterpenoid	1684
77	31.575	3.29			juniper camphor	bicyclic sesquiterpenoid	1691
		88.10%	97.17%	88.85%			
		34 cpd	57 cpd	47 cpd			



Chromatogram 4. Comparison of all three methods of analysis - sample **LOH LL4** chemotype. Counts vs. Acquisition Time (min).

A comparison of the number of identified substances for the four different chemotypes by the three different methods can be found in the Table 5 and results of quantitative determination of compounds for which we had commercially available standards are in Table 6.

Table 5. Number of identified compounds in different samples by three different methods and all identified different compounds in each chemotype from all three methods.

GC/MS	LOH LL1	LOH LL2	LOH LL3	LOH LL4
Liquid	37	38	45	34
HS	51	74	55	57
SPME	46	57	49	47
all identified different compounds	67	94	84	77

Table 6. Quantitative determination of terpenes/terpenoids for which were commercially available standards.

Compound	LOH LL1	LOH LL2	LOH LL3	LOH LL4
	µg/g	µg/g	µg/g	µg/g
α-pinene	45.6	60.0	101.6	47.0
camphene	13.5	18.33	29.38	14.4
β-pinene	83.7	102.0	147.5	62.0
β-myrcene	2136.2	294.3	744.1	544.2
limonene	473.3	705.9	803.7	457.1

 doi:10.20944/preprints202406.0373.v1

linalool		151.5	236.1	367.6	231.3
β-caryophyllene		1322.1	1704.0	542.1	1599.4
α-humulene		535.0	538.4	152.1	605.2
caryophyllene oxide	0.1		0.2	0.2	0.2
guaiol	-		313.7	288.8	522.3
α-bisabolol	-		-	170.0	64.7

4. Discussion

Gas chromatography analysis of the essential oil from Cannabis sativa was published already in 1957 [30]. In oil from fresh large leaves of female Cannabis sativa the compounds identified by gas chromatography were myrcene, limonene, α-humulene, and β-caryophyllene [31]. By steam distillation of fresh Indian Cannabis sativa L. from Kashmir 21 terpenes/terpenoids were identified in the essential oil [32].

Static headspace gas chromatography has already been used for marijuana and hashish analysis by Hood et al. [33,34]. They identified 16 terpenes and 1 terpenoid in the samples. For simultaneous quantification of 93 terpenoids present in air-dried Cannabis inflorescences and extracts static headspace – GC/MS/MS was used [35]. We also used GC/MS for identification of volatiles in different chemotypes of cannabis (medical and industrial) and published content volatiles, mostly terpenes/terpenoids, and their ratios in cannabis inflorescences and essential oils [36]. Thirteen chemotypes with different main terpenes/terpenoids were presented.

Solid-phase microextraction GC/MS was used to identify cannabidiol, Δ^8-tetrahydrocannabinol, Δ^9-tetrahydrocannabinol, and cannabinol in pure water and human saliva [37]. For cannabinoids determination in cannabis samples GC/MS method was developed [38]. Yang et al. [39] identified 13 monoterpenes, 4 monoterpenoids, and 14 sesquiterpenes in cannabis essential oil. The three mentioned gas chromatography techniques (HS, SPME, and liquid injection) were compared [40]. All three were excellent for the lower boiling monoterpenes. In HS sesquiterpenes were underrepresented. SPME gave a stronger signal for early eluting sesquiterpenes. Higher boiling sesquiterpenes were only adequately represented in liquid injection (hexane extract). Myers et al. [41] compared headspace-syringe and liquid injection-syringe techniques to the more modern headspace-solid phase microextraction arrow and direct immersion-SPME arrow. They used 23 terpene/terpenoids standards and determined from the results that the liquid injection-syringe method is the most straightforward and robust method.

We compared three different gas chromatography/mass spectrometry methods - static head-space extraction, head-space solid phase microextraction, and hexane based liquid extraction - to identify volatile compounds in cannabis samples, mainly monoterpenes/monoterpenoids and sesquiterpenes/sesquiterpenoids. We found hexane to be the best solvent for analysis of a liquid samples. Liquid samples give the most complex spectrum of the main mono- and sesquiterpenes/terpenoids as sesquiterpenes/sesquiterpenoids can be seen with higher retention times. Such liquid samples also have the advantage for absolute quantification of terpenes. The static headspace chromatogram gives the best representation of monoterpenes and monoterpenoids but a weaker signal for sesquiterpenes and sesquiterpenoids. Solid-phase microextraction gives significant spectrum of sesquiterpenes and sesquiterpenoids with shorter retention times and weaker signal for monoterpenes and monoterpenoids.

It seems that the results of Liq and SPME are the most similar (but not in all cases), so the analysis of the extract prepared with an organic solvent (hexane) will be the most suitable for the quantitative determination of these substances.

Altogether 26 terpenes/terpenoids were between the ten main present in four different chemotypes. They were divided with Liq containing 16 terpenes/terpenoids (2 terpenes, 1 terpenoid, 9 sesquiterpenes, and 4 sesquiterpenoids), 15 in HS (5 terpens, 2 terpenoids, 8 sesquiterpenes) and 17 in SPME (4 terpenes, 3 terpenoids, 10 sesquiterpenoids). The main terpene in chemotype LOH LL3 from the HS and SPME methods was limonene, but as we can see from Table 3 the other terpenes/terpenoids do not follow the same relative ratio. Chemotype LOH LL4 has a similar situation. The main terpene in Liq and SPME is β-caryophyllene, but as we can see from Table 4 the

 doi:10.20944/preprints202406.0373.v1

other terpenes/terpenoids do not follow the same relative ratio. As we can see from Tables 1 to 4, the terpenes found most often from amongst the ten main were β-myrcene, limonene, β-caryophyllene, α-humulene, γ-selinene, and selina-3,7(11)-diene. The most reproducible analysis for quantitative analysis appears to be from Liq samples.

It is now well known that different cannabis chemotypes (also mentioned as variety, strain, chemovar, cultivar, phenotype, or genotype) with the same content of major cannabinoids act differently in the treatment of the same patient. Not every chemotype is suitable for a given patient. Cannabis constituents (whether cannabinoids, terpenes/terpenoids or the other bioactive substances) interact with each other and can thus increase (or decrease) the effectiveness of a given chemotype [16,42]. If these effects are independent, synergistic or entourage, they still need to be thoroughly studied. In addition, each of us is genetically different and therefore one chemotype that is suitable for one patient might not be suitable for another one. It should also be emphasized that a given chemotype of cannabis that is suitable for a particular patient in a given disease may not (but sometimes may) cure a different disease.

5. Conclusions

In conclusion we can say that for the final evaluation of the comparison of the three methods of analysis, extraction with hexane gives balanced results (which is advantageous for quantitative analysis), although the other two methods allowed for the identification of more substances. This means that the same method should be used everywhere for the quantitative evaluation of constituents in cannabis. Only in this way it will be possible to objectively compare the results from different laboratories. The differences for the same sample analyzed in different laboratories will then be within the allowable error range.

Acknowledgments: My thanks to Margalit Lillie Beck for manuscript reading and language corrections.

Statement of Ethics: The author has no ethical conflicts to disclose.

Conflicts of Interest Statement: The authors have no conflicts of interest to declare.

Funding Sources: There were no external funding sources to the study in the preparation of data or the manuscript.

Abbreviations

Liq: hexane based liquid extraction; HS: static head-space extraction; SPME: head-space solid phase microextraction; Key: RT = retention time, % norm = % of the given peak relative to the main peak (main peak = 100 %), RI = retention index

References

1. Booth, J.K., Page, J.E., Bohlmann, J. Terpene synthases from Cannabis sativa, PLoS ONE. 2017; 12(3): e0173911.
2. Booth, J. Terpene and isoprenoid biosynthesis in Cannabis sativa. PhD thesis, The University of British Columbia, Vancouver, Canada, 2020; 223 pages.
3. Zhao, L., Chang, W., Xiao, Y., Liu, H., Liu, P. Methylerythritol Phosphate Pathway of Isoprenoid Biosynthesis. Ann Rev Biochem 2013; 82: 497–530.
4. Lichtenthaler, H.K. The plants' 1-deoxy-D-xylulose-5-phosphate pathway for biosynthesis of isoprenoids, Fett/Lipid 1998; 100 (4-5): 128–138.
5. Lichtenthaler, H.K. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants, Ann. Rev. Plant Physiol. Plant Mol. Biol. 1999; 50: 47–65.
6. Mechoulam, R., Gaoni, Y. The isolation and structure of cannabinolic, cannabidiolic and cannabigerolic acids, Tetrahedron 1965; 21: 1223-1229.
7. Fellermeier, M., Zenk, M.H. Prenylation of olivetolate by a hemp transferase yields cannabigerolic acid, the precursor of tetrahydrocannabinol, FEBS Letters 1998; 427: 283-285.
8. Rohmer, M. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants, Nat Prod Rep. 1999; 16: 565–574.
9. Baron, E.P. Medicinal Properties of Cannabinoids, Terpenes, and Flavonoids in Cannabis, and Benefits in Migraine, Headache, and Pain: An Update on Current Evidence and Cannabis Science, Headache 2018; 58(7): 1139-1186.

 doi:10.20944/preprints202406.0373.v1

10. Tomko, A.M., Whynot, E.G/, Ellis, L.D., Dupré, D.J. Anti-Cancer Potential of Cannabinoids, Terpenes, and Flavonoids Present in Cannabis, Cancers 2020; 12(7): 1985.
11. Kumar P, Mahato DK, Kamle M, Borah R, Sharma B, Pandhi S, Tripathi V, Yadav HS, Devi S, Patil U , Xiao J, Mishra AK. Pharmacological properties, therapeutic potential, and legal status of Cannabis sativa L.: An overview. Phytother. Res. 2021; 35: 6010-29.
12. Liktor-Busa E, Keresztes A, LaVigne J, Streicher JM, Largent-Milnes TM. Analgesic Potential of Terpenes Derived from Cannabis sativa. Pharmacol. Rev. 2021; 73: 1269–97.
13. Laws III JS, Shrestha S, Smid SD. Cannabis terpenes display variable protective and anti-aggregatory actions against neurotoxic β amyloid in vitro: highlighting the protective bioactivity of α-bisabolol in motorneuronal-like NSC-34 cells. Neurotoxicology 2022; 90: 81–87.
14. Rodriguez CEB, Ouyang L, Kandasamy R. Antinociceptive effects of minor cannabinoids, terpenes and flavonoids in Cannabis. Behav. Pharmacol. 2022; 33: 130-157.
15. Di Sotto A|, Gullì M, Acquaviva A, Tacchini M, Di Simone SC, Chiavaroli A, Recinella L, Leone S, Brunetti L, Orlando G, Flores GA, Venanzoni R, Angelini P, Menghini L, Ferrante C. Phytochemical and pharmacological profiles of the essential oil from the inflorescences of the Cannabis sativa L. Industrial Crops & Products 2022; 183: 114980.
16. Russo EB. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Br J Pharmacol. 2011; 163(7): 1344-64.
17. Blasco-Benito S, Seijo-Vila M, Caro-Villalobos M, Tundidor I, Andradas C, Garcia-Taboada E, Wade J, Smith S, Guzmán M, Pérez-Gómez E, Gordon M, Sanchez C. Appraising the "entourage effect": Antitumor action of a pure cannabinoid versus a botanical drug preparation in preclinical models of breast cancer. Biochem Pharmacol. 2018; 157: 285-93.
18. Worth T.Unpicking the entourage effect. Nature 2019; 572(7771): S12-S13.
19. Santiago M, Sachdev S, Arnold JC, McGregor IS, Connor M. Absence of entourage: terpenoids commonly found in *Cannabis sativa* do not modulate the functional activity of Δ9-THC at human CB1 and CB2 receptors. Cannabis and Cannabinoid Research 2019; 4(3): 165-176.
20. Finlay DB, Sircombe KJ, Nimick M, Jones C, Glass M. Terpenoids from cannabis do not mediate an entourage effect by acting at cannabinoid receptors. Front Pharmacol. 2020; 11: 359.
21. Ferber SG, Namdar D, Hen-Shoval D, Eger G, Koltai H, Shoval G, Shbiro L, Weller A. The "Entourage Effect": Terpenes Coupled with Cannabinoids for the Treatment of Mood Disorders and Anxiety Disorders. Curr Neuropharmacol. 2020; 18(2): 87-96.
22. LaVigne JE, Hecksel R, Keresztes A, Streicher JM. Cannabis sativa terpenes are cannabimimetic and selectively enhance cannabinoid activity. Sci Rep 2021; 11(1): 8232.
23. D'Amour FE, Smith Donn L. A method for determining loss of pain sensation. Journal of Pharmacology and Experimental Therapeutics 1941; 72(1): 74-9.
24. Dewey WL, Harris LS, Howes JF, Nuite JA. The effect of various neurohumoral modulators on the activity of morphine and the narcotic antagonists in the tail-flick and phenylquinone tests. Journal of pharmacology and experimental therapeutics 1970; 175(2): 435-42.
25. Pertwee RG. The ring test: a quantitative method for assessing the 'cataleptic' effect of cannabis in mice. Br J Pharmacol. 1972; 46(4): 753-63.
26. Little PJ, Compton DR, Johnson MR, Melvin LS, Martin BR. Pharmacology and stereoselectivity of structurally novel cannabinoids in mice. J Pharmacol Exp Ther. 1988; 247(3): 1046-51.
27. Turek C, Stintzing FC. Stability of essential oils: a review. Comprehensive Reviews in Food Science and Food Safety 2013; 12(1): 40-53.
28. Bueno J, Leuer E, Kearney M Jr, Green EH, Greenbaum EA. The preservation and augmentation of volatile terpenes in cannabis inflorescence. J Cannabis Res. 2020; 2(1): 27.
29. Prakash Chanda Gupta. Method Validation of Analytical Procedures. PharmaTutor Magazine 3(1), 32-39 (2015)
30. Stahl Egon: Chemical varieties of plants containing terpenoids. Essenze e Derivati Agrumari 1957; 27: 188-220.
31. Martin L, Smith DM, Farmilo CG. Essential oil from fresh Cannabis sativa and its use in identification. Nature 1961; 191: 774-76.
32. Nigam MC, Handa KL, Nigam IC, Levi L. Essential oils and their constituents XXIX. The essential oil of marihuana: composition of genuine Indian Cannabis sativa L. Can J Chem. 1965; 43(12): 3372-76.
33. Hood LVS, Dames ME, Barry GT. Headspace volatiles of marijuana. Nature 1973; 242(5397): 402-3.
34. Hood LVS, Barry GT. Headspace volatiles of marihuana and hashish: gas chromatographic analysis of samples of different geographic origin. J Chromatogr. 1978; 166(2): 499-506.
35. Shapira A, Berman P, Futoran K, Guberman O, Meiri D. Tandem Mass Spectrometric Quantification of 93 Terpenoids in Cannabis Using Static Headspace Injections. Anal Chem. 2019; 91(17): 11425-32.
36. Hanuš LO, Hod Y. Terpenes/terpenoids in Cannabis – are they important? Medical Cannabis and Cannabinoids 2020; 3: 25-60.

 doi:10.20944/preprints202406.0373.v1

37. Hall BJ, Satterfield-Doerr M, Parikh A., Brodbelt JS. Determination of Cannabinoids in Water and Human Saliva by Solid-Phase Microextraction and Quadrupole Ion Trap Gas Chromatography/Mass Spectrometry. Anal Chem. 1998; 70(9): 1788-96.
38. Ilias Y, Rudaz S, Mathieu P, Christen P, Veuthey JL. Extraction and analysis of different Cannabis samples by headspace solid-phase microextraction combined with gas chromatography-mass spectrometry. J Sep Sci. 2005; 28: 2293–2300.
39. Yang Zaibo, Long Chengmei, Zhong Caining, Sun Chengbin, Mao Haili: Analysis of chemical constituents of the volatile oil from Cannabis sativa by SPME-GC-MS. Zhongguo Yao Fang (Journal of China Pharmacy) 2008; 19(33): 2613-14.
40. Krill C, Rochfort S, Spangenberg G. A High-Throughput Method for the Comprehensive Analysis of Terpenes and Terpenoids in Medicinal Cannabis Biomass. Metabolites 2020; 10: 276.
41. Myers C, Herrington JS, Hamrah P, Anderson K. Accelerated Solvent Extraction of Terpenes in Cannabis Coupled With Various Injection Techniques for GC-MS Analysis. Front Chem. 2021; 9: 619770.
42. Russo, EB. The Case for the Entourage Effect and Conventional Breeding of Clinical Cannabis: No "Strain," No Gain. Frontiers in plant science 2018; 9: 1969.