Exploring the Chemical Space around Cannabis sativa L. Leaves as a Source of Bioactive Compounds of Pharmaceutical Interest

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Abstract

Cannabis sativa L. is a plant with a complex chemical profile of secondary metabolites, well established especially for what concerns inflorescences. Other parts of the plant, such as the leaves, are usually considered a waste material from the hemp industry and, therefore, are not yet considered in the research field. In this study, the leaves of four non-psychotropic *C. sativa* (hemp) varieties, including a CBD-, a CBG-, a CBC-type, and a cannabinoid-free one, were comprehensively characterized for their qualitative and quantitative composition of polyphenols, cannabinoids, policosanols, and terpenes. In addition, the present work also aimed at the development of an extraction and analytical method for quantification of squalene from the leaves of the four hemp chemotypes, in the perspective of finding an alternative plant source to recover this compound. Analytical techniques applied included UHPLC-HRMS and GC-MS for compound identification, while HPLC-UV/Vis, HPLC-ELSD, and GC-FID for quantification purposes. Cannflavin A and B were the predominant non-cannabinoid phenolic compound in the leaves. Among cannabinoids, cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), and cannabichromenic acid (CBCA) were the most abundant compounds in the analyzed samples. Minor CBD-type and CBG-type cannabinoids were detected in the leaves. The analysis of policosanols was focused on C_{24}OH, C_{26}OH, C_{28}OH, C_{30}OH, and C_{32}OH as the main compounds. Squalene was isolated, fully characterized for the first time from hemp leaves, and it was identified and quantified by using GC-MS and GC-FID techniques. Terpenes were analyzed by GC-MS; in addition to those commonly found in *C. sativa* (i.e., β-caryophyllene, α-humulene, α-bisabolol and caryophyllene oxide), some volatile constituents specific for the varieties were also detected.

Overall, hemp leaves represent a rich source of bioactive compounds that could be exploited in the pharmaceutical field from a circular economy perspective. This study, in addition, gives new insights into the possibility of hemp as a potential plant-based alternative source of squalene.

Introduction

Cannabis sativa L. is an annual, mostly dioecious plant belonging to the *Cannabaceae* family [1]. It has a very complex chemical profile, containing several biologically active secondary metabolites from various chemical classes [2–4]. Among them, cannabinoids (CBs) are plant-specific bioactive compounds with a distribution that varies significantly among different plant organs, being strongly influenced by both abiotic and biotic factors [1,2]. CBs are predominantly produced in glandular trichomes of the plant, which are particularly abundant in the flowers and upper leaves [5], explaining the scientific interest mainly focused on plant inflorescences. CBs are originally biosynthesized in their acid form, which undergoes a spontaneous non-enzymatic decarboxylation leading to the formation of their corresponding neutral counterpart [3]. Based on the CBs composition [6], five different chemotypes can be recognized for *C. sativa*: chemotype I (D^9-tetrahydrocannabinol (D^9-THC)-rich), chemotype II (D^9-THC and cannabidiol (CBD) in similar amount), chemotype III (CBD-rich, with a content of D^9-THC < 0.3%, according to European regulation [7]), chemotype IV (cannabigerol (CBG)-rich) and chemotype V (CB-free). To date, only

a few studies have described plant varieties with a higher cannabichromene (CBC) content in the CB fraction [8]. CBs have been widely studied for their biological properties, with a peculiar attention on the main four D[9]-THC, CBD, CBG, and CBC, even if also minor CBs have been investigated for their potential pharmaceutical properties [9–13]. Among non-psychotropic CBs, CBD has been deeply investigated for its antiproliferative activity[14]. Minor CBs have recently shown an interesting antiproliferative activity as well [15].

Non-cannabinoid phenolic compounds, such as cannflavins and canniprene, have been identified as another class of relevant secondary metabolites in *C. sativa,* and they have been mainly described for their antioxidant activity [16,17]. Cannflavin A (CFL-A), cannflavin B (CFL-B), and their corresponding demethoxy derivatives, including demethoxy cannflavin A (demethoxy CFL-A) and demethoxy cannflavin B (demethoxy CFL-B), are flavones specifically biosynthesized in this plant [18] whereas canniprene is a characteristic prenylated bibenzyl compound [17]. Besides these, other phenolics have been detected, including phenolic acids, hydroxycinnamic acids, and phenolic amides [19,20].

Policosanols (PCs) are a class of non-polar bioactive compounds found in *C. sativa* [21]. They are a mixture of long-chain aliphatic alcohols, with a carbon chain typically ranging from 20 to 36 carbon atoms [22], which have demonstrated antioxidant and anti-inflammatory activities [21]. According to the literature, PCs are usually present in very low amounts as such [22], as they predominantly occur in the esterified form, resulting in a difficult extraction process with a low yield. For this reason, specific methods have been developed for the extraction of PCs, involving the cleavage of the ester bond with the formation of free fatty alcohols [23]. Another lipophilic compound previously cited in *C. sativa* is squalene [24]. Among unsaponifiable compounds, squalene is a polyunsaturated triterpene ($C_{30}H_{50}$), which is currently extracted from animal sources (i.e., shark liver oil) and widely employed in the pharmaceutical industry as an emulsion-based adjuvant for vaccine delivery [25]. Due to marine environment preservation concerns and related ethical issues [26], current research is focusing on the identification of plant-based squalene alternative sources. Currently, *Amaranthus caudatus* L. is known to be the plant with the highest content of squalene [27]. Previous studies focused on *C. sativa* composition have determined the presence of squalene in a threshing residue by Soxhlet extraction together with many volatile terpenes, including b-caryophyllene and a-humulene [24].

Terpenes give the characteristic aroma to *C. sativa*, they are quite abundant in the inflorescences and less represented in leaves. Some of them are cultivar-specific, while other, such as b-caryophyllene, are widely distributed in the plant [24].

As anticipated before, *C. sativa* chemical composition is well established for what concerns inflorescences; however, other parts of the plants, such as leaves, are usually considered as a waste material from the hemp industry and, therefore, they are less investigated for their content of bioactive compounds. In this study, the leaves of four hemp varieties, including a CBD-rich, a CBG-rich, a CBC-rich,

and a CB-free one, were comprehensively characterized for the first time, with their qualitative and quantitative composition of polyphenols, CBs, PCs, and terpenes. In addition, the present work also aimed at the isolation, chemical characterization, and quantification of squalene from the leaves of these varieties as a potential plant-based alternative source of this compound. Analytical techniques applied in this study included UHPLC-HRMS and GC-MS for compound identification, whereas HPLC-UV/Vis, HPLC-ELSD and GC-FID were employed for quantification purposes. Overall, hemp leaves could represent a rich source of bioactive compounds that could be exploited in the pharmaceutical ambit from a circular economy perspective.

Results

Chemical characterization and quantification of polyphenols in hemp leaves. All the samples were analyzed by means of the UHPLC-HRMS technique to obtain the qualitative composition of polyphenols in the samples. Compound identification was achieved by comparison of the retention time (t_R), precursor ion, and fragmentation pattern with those of the corresponding analytical standards when available, as for canniprene, cannflavin-A (CFL-A) and cannflavin-B (CFL-B). The other compounds were putatively determined by comparing the experimental data, including t_R, precursor and product ions, with those available in the literature [16,28]. The list of compounds identified in the leaves, and the corresponding mass spectral data, are shown in Table 1, while their occurrence in the samples is shown in the Supplementary Information (SI, Table S1).

The UHPLC-HRMS chromatograms of all the samples analyzed showed the presence of dihydroferulic acid, hydroxygallic acid and several phenolic amides. In addition to CFL-B and CFL-A, their corresponding demethoxy derivatives, namely demethoxy CFL-B and demethoxy CFL-A, were found in each sample analyzed. CFL-C and other cannflavin derivatives were only putatively identified on the basis of the data available in the literature [16].

Table 1. UHPLC-HRMS data of polyphenols detected in the extracts from hemp leaves, both in the positive and negative ion mode, together with their fragmentation data

Compound	t_R	$[M + H]^+$	MS/MS	$[M-H]^-$	MS/MS
N-Feruloyloctopamine	17.8	–	–	328.1127	310.1083 (53), 295.0846 (17), 161.0232 (100), 133.0520 (76)
Dihydroferulic acid	19.5	197.1172	197.1168 (100), 179.1063 (99), 161.0957 (26), 135.1166 (54), 133.1009 (43), 107.0856 (44)	–	–
Hydroxygallic acid	21.8	–	–	187.0965	187.0963 (65), 125.0958 (100), 97.0645 (17)
N-Coumaroyltyramine	22.5	284.1281	284.1272 (16), 147.0437 (100), 121.0646 (40), 119.0489 (10)	–	–
N-*cis*-Feruloyltyramine	23.3	314.1385	314.1375 (21), 177.0542 (100), 145.0280 (35), 121.0647 (39), 117.0334 (7)	312.1237	312.1238 (71), 297.1003 (21), 190.0499 (30), 178.0499 (52), 148.0517 (100), 135.0439 (39)
N-Coumaroyltyramine	24.1	284.1281	284.1272 (16), 147.0437 (100),	–	–

Table 1. UHPLC-HRMS data of polyphenols detected in the extracts from hemp leaves, both in the positive and negative ion mode, together with their fragmentation data

			121.0646 (40), 119.0489 (10)		
N-trans-Feruloyltyramine	24.9	314.1385	314.1375 (21), 177.0542 (100), 145.0280 (35), 121.0647 (39), 117.0334 (7)	312.1237	312.1238 (71), 297.1003 (21), 190.0499 (30), 178.0499 (52), 148.0517 (100), 135.0439 (39)
Apigenin	29.4	271.0601	271.0593 (100), 153.0179 (6), 147.0440 (1)	269.0452	269.0452 (100), 151.0025 (17), 149.0232 (12), 117.0332 (68), 107.0124 (13), 65.0018 (14)
Diosmetin/chrysoeriol	30.1	301.0706	301.0698 (100), 286.0463 (33), 258.0515 (12)	299.0557	299.0557 (46), 284.0322 (100), 256.0374 (26), 227.0340 (4), 107.0122 (5), 63.0225 (8)
Cannflavin derivative	34.5	453.1909	453.1895 (12), 435.1791 (4), 313.0697 (100), 298.0463 (12)	451.1761	451.1757 (100), 309.0401 (36), 297.0373 (24), 163.0026 (50), 133.0281 (26)
Cannflavin derivative	34.7	401.1232	367.1165 (47), 325.0697 (100), 313.0696 (91),	–	–

Table 1. UHPLC-HRMS data of polyphenols detected in the extracts from hemp leaves, both in the positive and negative ion mode, together with their fragmentation data

			310.0463 (35), 297.0749 (42)		
Acacetin	35.9	285.0757	285.0749 (100), 285.110 (39), 270.0516 (12), 242.0564 (14	–	–
Isocannflavin B	37.2	369.1333	369.1319 (31), 313.0695 (100), 298.0458 (12)	367.1185	–
Cannflavin derivative	37.6	435.1804	435.1797 (3), 313.0696 (100), 298.0462 (13), 183.0284 (2), 165.0178 (7)	–	–
Cannflavin derivative	38.0	453.1909	453.1894 (5), 314.0730 (22), 313.0697 (100), 298.0462 (12), 165.0178 (7)	451.1761	451.1759 (100), 351.0973 (29), 309.0400 (51), 297.0401 (52), 163.0024 (10), 133.0281 (55)
Cannflavin derivative	38.4	469.1857	–	467.1707	435.1444 (100), 391.1180 (32), 311.0556 (20), 297.0398 (56), 163.0024 (19), 133.0281 (36)
Cannflavin derivative	38.6	469.1858	313.0696 (100), 298.0463 (13),	–	–

Table 1. UHPLC-HRMS data of polyphenols detected in the extracts from hemp leaves, both in the positive and negative ion mode, together with their fragmentation data

			183.0284 (2), 165.0178 (7)		
Demethoxy CFL-B	38.7	339.1277	–	337.1079	337.1074 (100), 293.0458 (12), 281.0453 (11), 161.0229 (9), 133.0643 (10), 117.0331 (63)
CFL-B	39.0	369.1332	369.1321 (6), 313.0696 (100), 298.0463 (16), 165.0178 (10)	367.1182	367.1180 (91), 352.0952 (43), 309.0399 (100), 297.0400 (32), 269.0451 (13), 133.0282 (50)
Canniprene	39.7	343.1889	343.1891 (4), 287.1268 (100), 255.1007 (44), 227.1059 (24)	341.1758	341.1751 (100), 326.1517 (20), 283.0977 (13), 269.0918 (5)
CFL-C	41.1	437.1960	437.1947 (66), 313.0696 (100), 298.0462 (17)	435.1810	435.1806 (100), 420.1583 (29), 351.0862 (36), 297.0398 (42), 268.0382 (15), 133.0282 (34)
Demethoxy CFL-A	42.8	407.1854	407.1838 (7), 283.0591 (100), 183.0282 (2), 165.0177 (11)	405.1706	405.1702 (100), 293.0451 (32), 281.0452 (77), 163.0025 (26),

Table 1. UHPLC-HRMS data of polyphenols detected in the extracts from hemp leaves, both in the positive and negative ion mode, together with their fragmentation data					
					161.0232 (33), 117.0332 (33)
CFL-A	43.1	437.1960	437.1947 (66), 313.0696 (100), 298.0462 (17)	435.1808	435.1806 (100), 420.1574 (40), 351.0866 (36), 309.0404 (19), 297.0388 (15), 133.0281 (30)

Quantitative data of polyphenols in hemp leaves were obtained by HPLC-UV/Vis. Chromatograms were recorded at 342 nm for cannflavins and at 210 nm for canniprene. A representative chromatogram is shown in Fig. 1. CFL-A and CFL-B were quantified by means of a calibration with the corresponding reference compound, while demethoxy CFL-A and demethoxy CFL-B were quantified by using the calibration curve of CFL-A and CFL-B, respectively. As shown in Table 2, CFL-A was found in a higher amount than CFL-B in the samples analyzed in this work. Demethoxy CFL-A and CFL-B were abundant mostly in sample 1. Canniprene amount exceeded the limit of quantification (LOQ) in sample 3 only.

Table 2
Quantitative analysis of polyphenols by HPLC–UV/Vis, expressed as g/g ± standard deviation (SD, n = 4)

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Compound	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
Demethoxy CFL-B	28.1 ± 0.6	< LOQ	< LOQ	6.9 ± 0.4
Canniprene	–	< LOQ	23.8 ± 0.7	< LOQ
CFL-B	33.6 ± 0.6	11.0 ± 0.5	10.3*	17.6 ± 0.3
Demethoxy CFL-A	41.7 ± 0.5	< LOQ	< LOQ	< LOQ
CFL-A	70.1 ± 1.8	26.3 ± 1.9	20.9 ± 0.5	174.7 ± 2.5
* SD < 0.05				

Chemical characterization and quantification of CBs in hemp leaves. An untargeted analysis of CBs in the extracts of hemp leaves belonging to different varieties was carried by means of UHPLC-HRMS. The complete characterization was performed by comparing the experimental data of compounds (t_R, precursor and product ions) with those of analytical standards and available data in the literature. The

acidic and neutral CBs detected in the samples are shown in Tables 3 and 4, while their distribution is available in the SI (Tables S2 and S3). Tables 3 and 4 include also data from CBDA esters, CBG-type and CBN-type compounds, being them investigated here for the first time. For these compounds, the reported mass spectrometric data refer to the corresponding analytical pure standards.

Table 3
UHPLC-HRMS data of CBs investigated in the extracts from hemp leaves, both in the positive and negative ion mode, together with their fragmentation data

Compound	t_R	$[M+H]^+$	MS/MS	$[M-H]^-$	MS/MS
Cannabinodiolic acid (CBNDA)	7.0	–	–	353.1757	309.1859 (100), 279.1388 (29), 171.0805 (32)
Cannabidivarinic acid (CBDVA)	8.1	331.1799	313.1799 (100), 233.1175 (12), 191.0704 (36)	329.1759	311.1653 (71), 285.1859 (20), 217.1230 (60), 151.0755 (33)
Cannabidibutolic acid (CBDBA)	9.6	345.2068	327.1965 (100), 247.1332 (13), 205.0861 (38)	343.1914	343.1914 (100), 325.1809 (67), 299.2016 (27), 231.1388 (61), 165.0913 (33)
Cannabidiolic acid (CBDA)	10.8	359.2213	341.2114 (100), 261.1489 (10), 219.1017 (21), 135.0441 (4)	357.2073	357.2073 (100), 339.1965 (60), 313.2174 (24), 245.1545 (58), 179.1069 (31)
Cannabigerolic acid (CBGA)	11.1	–	–	359.2228	341.2121 (100), 359.2228 (48), 315.2328 (42), 191.1070 (13)
CBDA geraniol ester	11.7	–	–	–	–
Cannabidihexolic acid (CBDHA)	11.9	–	–	371.2233	371.2231 (100), 353.2125 (49), 327.2326 (23), 325.2169 (19), 259.1703 (57), 193.1226 (39)
Cannabidiphorolic acid (CBDPA)	12.8	–	–	385.2388	385.2388 (100), 367.2283 (56), 341.2495 (29), 339.2325 (22), 273.1863 (72), 207.1385 (39)
Tetrahydrocannabivarinic acid (THCVA)	13.0	331.1801	331.1801 (7), 313.1801 (100), 191.0705 (24)	329.1760	329.1760 (100), 285.1865 (65), 217.1228 (31), 163.0755 (28)
Cannabichromenvarinic acid (CBCVA)	13.5	–	–	329.1758	329.1758 (100), 311.1651 (28), 285.1858 (28), 215.1073 (14), 163.0755 (56)

Compound	t_R	$[M + H]^+$	MS/MS	$[M–H]^–$	MS/MS
CBDA borneol ester	13.5	495.3456	495.3490 (3), 341.2112 (59), 219.1017 (100)	–	–
Cannabinolic acid (CBNA)	14.1	–	–	353.1761	353.1761 (64), 309.1806 (100), 279.1391 (52), 171.0807 (19)
Tetrahydrocannabinoic acid (THCA)	15.4	359.2224	359.2224 (5), 341.2114 (100)	357.2072	357.2072 (100), 313.2174 (54), 245.1545 (24), 191.1070 (22)
CBDA nerol ester	15.4	–	–	–	–
CBDA fenchol ester	15.6	495.3455	495.3455 (70), 341.2120 (92), 81.0706 (100)	–	–
Cannabigerolic acid monomethyl ether (CBGMA)	16.1	–	–	373.2384	373.2385 (57), 329.2486 (60), 245.1546 (43), 191.1069 (100)
Cannabichromenic acid (CBCA)	16.2	359.2213	359.2219 (100), 341.2113 (85)	357.2076	357.2076 (100), 339.1966 (21), 313.2175 (20), 243.1395 (9), 191.1071 (50), 179.1070 (5)

Sample 1 exhibited the wider CB profile, including several minor CBs belonging to the CBD-type chemical class. Among them, cannabidihexolic acid (CBDHA), cannabidiphorolic acid (CBDPA), and their corresponding neutral counterparts, i.e., cannabidihexol (CBDH) and cannabidiphorol (CBDP), were putatively identified [5,29]. Moreover, other minor CBs were identified in this sample, including cannabinodiolic acid (CBNDA), tetrahydrocannabivarinic acid (THCVA), cannabichromenvarinic acid (CBCVA) and cannabigerolic acid monomethyl ether (CBGMA) among acidic CBs, and cannabigerophorol (CBGP), cannabicitran (CBTC), sesqui-cannabigerol (sesqui-CBG) and cannabigerohexol (CBGH) as neutral CBs. Only CBGP, having a $[M + H]^+$ precursor ion at 331.2626 *m*/*z* and two product ions at 221.1538 and 137.0598 *m*/*z*, and sesqui-CBG, having a $[M + H]^+$ precursor ion at 385.3102 *m*/*z* and two product ions at 193.1225 and 123.0443 *m*/*z*, were confirmed by comparison with reference standards, while the other compounds were putatively identified on the basis of the literature data [5,29]. Samples 2 and 3 exhibited a similar CB profile to sample 1, except for the absence of minor CBD-type compounds (CBDHA, CBDPA, CBDH and CBDP). Sample 4 was found to contain both acidic and neutral CBs, in particular CBNDA, cannabidivarinic acid (CBDVA), cannabidibutolic acid (CBDBA),

CBDA, CBGA, cannabinolic acid (CBNA), CBGMA, cannabichromenic acid (CBCA), CBG, CBD and CBTC, though their peaks had a much lower intensity with respect to the other samples.

None of the minor CBN-type cannabinoids, namely cannabinerovarin (CBNRV), cannabinerobutol (CBNRB), cannabinerol (CBNR) and cannabinerophorol (CBNRP), were detected in any of the four samples. Cannabinol (CBN) was detected only in samples 1 and 2. This is consistent with the fact that CBN is an oxidation product of Δ^9-THC [30], which was detected at very low levels in the samples analyzed in this work.

Additionally, some CBDA esters were also investigated in the leaves, including CBDA geraniol ester, CBDA borneol ester, CBDA nerol ester and CBDA fenchol ester. In general, CBDA esters had a $[M + H]^+$ precursor ion at 495.3456 *m*/*z*. Among these compounds, CBDA borneol ester, having two product ions at 341.2112 and 219.1017 *m*/*z*, was the only one identified in samples 1 and 2.

Table 4

UHPLC-HRMS data of neutral CBs investigated in the extracts from hemp leaves, both in the positive and negative ion mode, together with their fragmentation data

Compound	t_R	$[M+H]^+$	MS/MS	$[M-H]^-$	MS/MS
Cannabidivarin (CBDV)	8.9	287.2007	287.2007 (100), 231.1383 (18), 165.0912 (52), 135.1171 (19), 123.0444 (9)	285.1861	–
Cannabinerovarin (CBNRV)	8.9	289.2161	289.2164 (4), 165.0912 (100), 123.0443 (7), 69.0707 (2)	–	–
Cannabigerovarin (CBGV)	8.9	289.2159	289.2160 (5), 165.0911 (100), 123.0443 (7), 69.0706 (29)	287.2019	287.2019 (100), 163.0758 (16), 151.0751 (7)
Cannabinerobutol (CBNRB)	10.2	303.2320	303.2320 (3), 179.1068 (100), 123.0443 (10)	–	–
Cannabigerobutol (CBGB)	10.3	303.2316	303.2318 (5), 179.1067 (100), 123.0443 (11)	–	–
Cannabidibutol (CBDB)	10.5	301.2163	301.2163 (100), 245.1539 (17), 179.1068 (47), 123.0443 (14), 93.0705 (19)	299.2016	299.2016 (92), 231.1388 (100), 165.0911 (60), 107.0490 (85)
Cannabinerol (CBNR)	11.3	317.2473	317.2475 (4), 193.1226 (100), 137.0599 (2), 123.0444 (14)	–	–
Cannabigerol (CBG)	11.4	317.2477	317.2477 (4), 193.1225 (100), 137.0599 (4), 123.0443 (12)	–	–
Cannabidiol (CBD)	11.6	315.2321	315.2321 (100), 259.1694 (17), 193.1225 (55), 135.1170 (20), 123.0444 (19), 93.0704 (21)	313.2173	313.2173 (79), 245.1545 (100), 179.1069 (49)

Compound	t_R	$[M + H]^+$	MS/MS	$[M–H]^–$	MS/MS
Tetrahydrocannabivarin (THCV)	11.8	287.2007	287.2007 (100), 231.1383 (18), 165.0912 (54), 135.1170 (19), 123.0444 (10)	–	–
Cannabidihexol (CBDH)	12.6	329.2475	329.2481 (52), 273.1489 (23), 207.1382 (28), 135.1170 (16), 93.0705 (39)	–	–
Cannabinerophorol (CBNRP)	13.0	345.2787	345.2788 (3), 221.1536 (100), 123.0443 (13)	–	–
Cannabigerophorol (CBGP)	13.1	345.2787	345.2787 (4), 221.1538 (100), 137.0598 (7), 123.0443 (18)	–	–
Cannabinol (CBN)	13.2	311.2007	311.2007 (100), 293.1900 (26), 241.1226 (16), 223.1119 (55)	309.186	309.1860 (100), 279.1390 (23), 171.0806 (6)
Cannabidiphorol (CBDP)	13.5	343.2632	343.2628 (100), 287.2002 (17), 221.1537 (36), 135.1170 (23), 93.0704 (25)	–	–
Cannabicitran (CBTC)	13.7	315.2319	315.2319 (100), 259.1693 (15), 193.1225 (41), 135.1169 (17), 93.9704 (16)	–	–
Δ^9-Tetrahydrocannabinol (Δ^9-THC)	14.2	315.2319	315.2319 (100), 259.1695 (17), 193.1225 (43), 135.1169 (21), 123.0443 (16)	–	–
Sesqui-cannabigerol (sesqui-CBG)	14.7	385.3102	385.3102 (3), 193.1225 (100), 123.0443 (12)	383.2960	383.2960 (100), 245.1540 (3), 191.1069 (15), 179.1065 (4)
Cannabichromene (CBC)	15.0	315.2322	315.2322 (34), 259.1696 (32), 193.1226 (100), 123.0444 (18)	–	–

Compound	t_R	$[M + H]^+$	MS/MS	$[M–H]^–$	MS/MS
Cannabigerohexol (CBGH)	15.9	331.2626	331.2629 (2), 207.1380 (100), 137.0597 (8)	–	–

Having completed the characterization of CBs, the next step involved the quantification of main compounds using HPLC-UV/Vis. Chromatograms were recorded at 220 nm and at 210 nm for acidic and neutral cannabinoids, respectively. A representative chromatogram from the HPLC-UV/Vis analysis of sample 1 is shown in Fig. 2. Table 5 shows the amount of acidic and neutral CBs quantified using HPLC-UV/Vis. Consistent with their declared origin, sample 1 was particularly rich in CBDA, while samples 2 and 3 had CBGA and CBCA as the main compounds, respectively. CBDA was quantified in sample 4, though at a low level compared to the other samples. Neutral CBs were present in lower content with respect to the corresponding acidic forms, having applied a sample preparation procedure that preserves the composition of CBs of the plant material.

Table 5. Quantitative analysis of acidic and neutral CBs by HPLC-UV/Vis, expressed as mg/g ± standard deviation (SD, $n = 4$).

Compound	Sample 1	Sample 2	Sample 3	Sample 4
CBDVA	<LOD	<LOD	–	<LOD
CBDV	<LOD	–	–	–
CBDBA	<LOQ	<LOD	<LOD	<LOD
CBDB	<LOQ	–	–	–
CBDA	43.0 ± 1.8	<LOQ	<LOQ	3.8 ± 0.8
CBGA	<LOQ	13.1 ± 0.3	4.4 ± 0.2	<LOD
CBG	<LOQ	1.8*	<LOQ	<LOD
CBD	1.2*	<LOQ	<LOQ	<LOQ
THCV	–	<LOD	<LOD	–
THCVA	<LOD	<LOD	–	–
CBN	<LOD	<LOD	–	–
CBNA	<LOD	<LOD	<LOD	<LOD
Δ^9-THC	<LOD	<LOD	<LOD	–
CBC	<LOQ	<LOQ	<LOQ	–
THCA	<LOQ	1.7*	5.4 ± 0.2	–
CBCA	<LOQ	<LOQ	12.8 ± 0.8	<LOD
* SD < 0.05				

Chemical characterization and quantification of lipophilic compounds in hemp leaves. The samples were analyzed by means of a validated HPLC-ELSD method for both the identification and quantification of PCs, including docosanol ($C_{22}OH$), tetracosanol ($C_{24}OH$), hexacosanol ($C_{26}OH$), octacosanol ($C_{28}OH$), triacontanol ($C_{30}OH$) and dotriacontanol ($C_{32}OH$) [21]. $C_{30}OH$ was quantified by means of a calibration with the corresponding reference compound, while $C_{32}OH$ was quantified by using the calibration curve of $C_{30}OH$. A representative HPLC-ELSD chromatogram of PCs in a hemp leaf extract is shown in Fig. 3.

Qualitative data related to the distribution of PCs in the samples are described in the SI (Table S4). Sample 4, i.e., the CB-free one, was found to contain all the PCs taken into consideration in this study. Only $C_{30}OH$ and $C_{32}OH$ were quantified in all the samples analyzed. Notably, samples 1 and 2 (CBD-type and CBG-type) exhibited a similar amount of $C_{30}OH$ (19.5±0.4 and 19.0±0.6 µg/g, respectively), whereas in samples 3 and 4 the content of this compound was below the LOQ. As for $C_{32}OH$, only sample 1 exhibited a quantifiable amount (20.1 ± 0.9 µg/g), while the concentration was below the LOQ in samples 2, 3, and 4.

Regarding squalene, the triterpenoid was first identified by isolation from a sample of hemp leaves with a series of vacuum filtrations to avoid waxes, fatty acids and CBs, using different stationary phases. The procedure led to obtain an enriched fraction of the metabolite (SI, Fig. S1), which was identified by comparison with the literature[31]. In the ^{1}H NMR analysis, the signal of the six olefinic protons (6H, δ_H 5.14, m), the presence of the ten methylene moieties (20H, δ_H 1.98–2.07, m) and the eight triterpenyl methyls (24H, δ_H 1.62–1.76, s) are visible.

Different extraction conditions were tested (data not shown), and the optimized one was used for the analysis of squalene in hemp leaves. Qualitative and quantitative analyses were carried out by means of GC-MS and GC-FID, respectively. Peak assignment was confirmed by comparing the t_R of the pure standard, analyzing samples spiked with the reference compound and using a MS spectral library search. GC-MS analysis of squalene in a hemp leaf extract revealed that the peak exhibited comparable spectral purity and the same fragmentation pattern as the reference standard, as shown in the SI (Figs. S2 and S3). A representative GC-FID chromatogram, highlighting the squalene elution window, is shown in Fig. 4. The content of squalene was relatively constant among the samples (12.9 ± 1.1 µg/g for sample 1, 11.0 ± 1.0 µg/g for sample 2, 14.7 ± 0.2 µg/g for sample 4), with a higher amount in sample 3 (29.2±1.4 µg/g).

Chemical characterization and quantification of terpenes in hemp leaves.

The analysis of the terpene fraction in the samples showed that each one has a distinctive profile of volatile compounds. A representative chromatogram from the GC-MS analysis of sample 1 is shown in Fig. 5. Terpenes detected in the samples are shown in Table 6, while their distribution is available in the SI (Table S5).

In detail, sample 4 has the lowest content of terpenes, while samples 1,2 and 3 have a total content of terpenes of around 1000 µg/g. In all samples, α-humulene, β-caryophyllene, α-bisabolol and caryophyllene oxide represent the most abundant terpenes. Valencene is found in significant amounts in samples 1 and 3, while farnesene is the most abundant terpene in samples 1 and 2. Other terpenes, such as camphene, limonene and β-myrcene, are more characteristic of one variety with respect to the others and are generally found in tenth of µg/g amounts. Eucalyptol is characteristic of sample 1, while camphene and borneol of sample 4. Ocimene was found only in sample 3, while sample 2 had no characteristic terpene.

Table 6. Quantitative analysis of terpenes by GC-MS, expressed as mg/g ± standard deviation (SD, n = 3).

Compound	Sample 1	Sample 2	Sample 3	Sample 4
α-Pinene	31.0 ± 4.0	<LOD	< LOD	6.0 ± 1.0
β-Myrcene	46.0 ± 6.0	6.0 ± 1.0	< LOD	7.0 ± 1.0
Camphene	< LOD	<LOD	< LOD	4.0 ± 0.5
Terpinolene	< LOD	<LOD	< LOD	3.1 ± 0.5
Limonene	25.0 ± 3.0	10.0 ± 2.0	<LOQ	5.3 ± 0.7
Fenchyl alcohol	29.0 ± 5.0	<LOD	<LOD	<LOQ
Borneol	< LOD	<LOD	<LOD	3.0 ± 0.4
β-Caryophyllene	420.0 ± 12.0	170.0 ± 10.0	700.0 ± 10.0	120.0 ± 5.0
α-Humulene	103.0 ± 9.0	46.0 ± 6.0	250.0 ± 8.0	20.0 ± 2.0
Caryophyllene Oxide	52.0 ± 4.0	50.0 ± 4.0	130.0 ± 9.0	60.0 ± 6.0
α-Bisabolol	895.0 ± 17.0	645.0 ± 13.0	30.0 ± 4.0	10.0 ± 2.0
Ocimene	< LOD	< LOD	70.0 ± 8.0	<LOD
trans-Nerolidol	< LOD	< LOD	20.0 ± 5.0	<LOD
cis-Nerolidol	405.0 ± 11.0	101.0 ± 10.0	90.0 ± 10.0	<LOD
Valencene	309.0 ± 10.0	<LOD	110.0 ± 1.0	<LOD
Guaiol	< LOD	173.0 ± 10.0	<LOD	<LOD
Cedrol	392.0 ± 12.0	198.0 ± 9.0	<LOD	<LOD
Farnesene	1035.0 ± 30.0	442.0 ± 12.0	<LOD	<LOD
Eucaliptol	27.0 ± 3.0	<LOD	<LOD	<LOD

Discussion

A comprehensive chemical characterization of bioactive compounds in non-psychotropic *C. sativa* leaves, belonging to different varieties, was performed for the first time in this study. The secondary metabolites considered included polyphenols, CBs, unsaponifiable lipids (PCs and squalene) and terpenes.

Polyphenols analysis established the ubiquitous presence of CFL-A and B in the samples, along with the corresponding demethoxy derivatives, confirming their role as relevant markers of this chemical class in hemp leaves [16,18]. Several studies have investigated these flavones, particularly in CBD-rich varieties in both leaves and inflorescences [20,32,33]. However, conflicting results were described in the literature for what concerns quantitative data. In the present study, the CFL-A amount in the CBD-type variety (sample

1, 70.1 ± 1.8 µg/g) was consistent with the results described in the literature for leaves of the same variety (69.95–76.11 µg/g) [33] and also for inflorescences (61.8 µg/g) [20]. In contrast, another study has described a significantly higher amount of CFL-A in *C. sativa* leaf extract (1369.1 µg/g), obtained under reflux extraction with methanol [32]. Interestingly, a higher CFL-A content was found in the CB-free variety (sample 4, 174.7 ± 2.5 µg/g), that may be considered as a promising source of this compound. Conversely, leaves from the CBG- and CBC-type varieties (samples 2 and 3) exhibited lower levels of CFL-A.

The CB analysis performed provided a detailed chemical fingerprint of *C. sativa* leaves. In addition to the main CBs, leaves from the CBD-type variety (sample 1) were found to contain minor compounds related to the CBD-type scaffold, namely CBDHA and CBDPA, together with their corresponding neutral counterparts CBDH and CBDP. Interestingly, CBGP and CBGH were identified in both CBD-type and CBG-type leaves (samples 1 and 2). Sesqui-CBG is ubiquitous among all leaf samples considered. Notably, the content of CBDA found in sample 1 (43.0 ± 1.8 mg/g) was on average comparable to the levels observed in the inflorescences from CBD-type varieties, i.e., ranging from 3.7 to 36.4 mg/g, as described in a previous study [4]. For what concerns CBGA, the highest content was found in sample 2 (13.1 ± 0.3 mg/g), which was from a CBG-type variety, in agreement with previous results on CBG-type inflorescences, having CBGA at 9.8 mg/g [3]. Conversely, CBCA levels were found to be higher in sample 3 (12.8 ± 0.8 mg/g), which were slightly lower than those described in the literature for a CBC-type variety biomass (22.0-28.4 mg/g) [8]. Very few CBs were detected in the CB-free leaves (sample 4), though none of them were quantifiable, with the exception of CBDA. In general, hemp leaves have been demonstrated to be a discrete rich source of CBs, and, therefore, they should be taken into consideration for the recovery of CBs as an alternative to commonly used inflorescences.

Concerning PCs, a wide range of these compounds was identified in the extracts of the leaves of all the varieties considered in this work, although only triacontanol ($C_{30}OH$) and dotriacontanol ($C_{32}OH$) were in a quantifiable amount. In general, hemp leaves exhibited a lower PCs content compared to inflorescences, with $C_{30}OH$ and $C_{32}OH$ levels ranging 21.6-141.2 µg/g and 23.1–96.2 µg/g [21], probably due to a lower content of waxy material. Noteworthily, *C. sativa* leaves were investigated for the first time here as a potential alternative “green” source of squalene, which was obtained and characterized in an enriched fraction of this triterpenoid. An appropriate extraction procedure and analytical method were also newly developed and optimized for this plant material. For the best outcome of the extraction, it was important to carefully select the best extraction technique, solvent, and time. Based on the analysis performed, ethyl acetate (EtOAc) was identified as the most efficient solvent for squalene extraction from hemp leaves using dynamic maceration for 4 h. The content of squalene determined in hemp leaves (from 11.0 ± 1.0 µg/g for sample 2 to 29.2±1.4 µg/g for sample 3) was higher in comparison to inflorescences from sample 3 itself (2.4±0.3 µg/g). Despite the content of squalene being lower with respect to a previously described Soxhlet extraction with heptane (60–160 µg/g, depending on the harvest stage) [34], the method developed in the present study enabled the extraction of this compound using a more “eco-friendly” procedure. With the development of appropriate enrichment and

fractionation procedures, *C. sativa* leaves could really represent a new source of this pharmaceutically relevant compound.

Regarding terpenes, variety-specific terpenes, such as eucalyptol and ocimene, were found in the considered samples, along with the ones commonly present in all strains (i.e., β-caryophyllene, α-humulene, α-bisabolol and caryophyllene oxide). Although some differences among the subvarieties analyzed can be observed, the overall similarity of their chemical profiles does not allow a clear differentiation between them, as previously described in the literature [35]. The observed variation in the composition and proportion of terpenes is consistent with known influences of both biotic and abiotic factors [35]. As expected, sesquiterpenes were predominant over monoterpenes [36].

Overall, *C. sativa* leaves, which are usually considered a waste product, demonstrated to be a diverse and rich source of bioactive compounds belonging to different chemical classes, paving the way to their possible exploitation in the pharmaceutical field in a concrete circular economy perspective.

Materials and Methods

Chemicals and reagents. Acetone, acetonitrile (ACN), chloroform ($CHCl_3$), cyclohexane, EtOAc, ethanol (EtOH), isopropanol, methyl tert-butyl ether (MTBE), *n*-hexane, petroleum ether (PE), formic acid (HCOOH) and potassium hydroxide (KOH) were purchased from Sigma-Aldrich (Milan, Italy), while ammonium formate was provided from Fluka (Charlotte, NC, USA). Water (H_2O) was purified using a Milli–Q® Advantage 10 system from Millipore (Milan, Italy). Basic alumina (50–75 μm), silica gel 60 (60–200 μm), silica gel for flash chromatography (50 μm), reversed-phase (RP) C_{18} silica gel (25 μm), and Celite® 545 (particle size 0.02–0.1 mm), used for low-pressure chromatography (LPC), flash chromatography, and vacuum chromatography, were purchased from Macherey-Nagel (Düren, Germany). Purifications were monitored by TLC on 60 F254 (0.25 mm) plates purchased from Merck (Darmstadt, Germany) visualized by staining with 5% H_2SO_4 in EtOH and heating. Cannflavin reference standards (CFL-A and CFL-B) were purchased from LGC standards (Milan, Italy), while canniprene (≥ 97%), isolated according to a procedure described in the literature [17], was kindly provided by Prof. Federica Pollastro. The standard solution of cannabidivarin (CBDV), cannabidibutolic acid (CBDBA), cannabidibutol (CBDB), cannabigerol (CBG), cannabidiol (CBD), tetrahydrocannabivarin (THCV), Δ^9-THC and CBC were purchased from LGC standards (Milan, Italy). A cannabinoic acids mixture, composed of cannabidivarinic acid (CBDVA), cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), tetrahydrocannabivarinic acid (THCVA), Δ^9-tetrahydrocannabinoic acid (THCA), cannabichromenic acid (CBCA), cannabinol (CBN) and cannabinolic acid (CBNA), was purchased from Merck Life Science s.r.l. (Milan, Italy). CBDA esters (≥ 97%), including CBDA geraniol ester, CBDA borneol ester, CBDA nerol ester and CBDA fenchol ester, obtained through a CBDA esterification procedure described in the literature [37], as well as minor CBG-type (≥ 97%) compounds, namely cannabigerovarin (CBGV), cannabigerobutol (CBGB), CBGP and sesqui-CBG and CBN-type (≥ 97%) compounds, namely cannabinerovarin (CBNRV), cannabinerobutol (CBNRB), cannabinerol (CBNR) and cannabinerophorol (CBNRP), synthesized following

a procedure previously described in the literature [15], were provided by Prof. Federica Pollastro. Reference policosanols, including docosanol (C_{22}OH, ≥ 97%), tetracosanol (C_{24}OH, ≥ 97%), hexacosanol (C_{26}OH, ≥ 95%), octacosanol (C_{28}OH, ≥ 95%) and triacontanol (C_{30}OH, ≥ 90%), were purchased from LGC standards (Milan, Italy). Squalene standard was purchased from TCI (Japan). Spex CertiPrep CAN-TERP-KIT-H Can-Terp Kit (42 components, 1.000 µg/mL) Kit was purchased from Cole-Parmer, England.

Hemp varieties investigated. Hemp plant material (leaves) from CBD-, CBG-, CBC-type and CB-free varieties were provided by Dr. Gianpaolo Grassi Canvasalus S.r.l. (Monselice, Italy). For each sample, the leaves were manually sieved before the extraction procedure. The samples were labelled as 1, 2, 3 and 4, corresponding to the CBD-, CBG-, CBC- and CB-type free variety, respectively. *C. sativa* leaves (industrial hemp variety Carmagnola) used for the isolation of squalene were provided by Dimensione Canapa (Cuccaro Monferrato, Italy) and collected in September 2024 (a reference standard named CarmgLeaves-DC/09/24 is stored in the laboratory of phytochemistry in Novara).

Extraction of polyphenols from hemp leaves. According to a previous work, 0.25 g of dried leaves was weighed and treated with 10 mL of *n*-hexane for 15 min under magnetic stirring to remove CBs. The washing procedure was repeated twice with 10 mL and 5 mL of *n*-hexane, respectively. After filtration with a paper filter, the filtrates were sent to waste, and the dried residue was extracted three times with 10 mL, 10 mL and 5 mL of acetone. The filtrates were collected and brought to dryness under vacuum with a rotary evaporator (Laborota 4000 Heidolph, Schwabach, Germany) and adjusted to a final volume of 1 mL with acetone. The solution was filtered through a 0.45 µm PTFE filter into an HPLC vial. The sample preparation was performed in duplicate for all the samples considered in this study. The samples were stored at − 20°C until analysis was performed [4].

Extraction of CBs from hemp leaves. A portion of 0.25 g of dried leaves was weighed and submitted to a dynamic maceration under magnetic stirring with 10 mL of EtOH for 15 min. The extract was filtered with a paper filter. The extraction was repeated twice with 10 mL and 5 mL of the extraction solvent, respectively. The extracts were pooled and adjusted to a final volume of 25 mL with EtOH. The solution was filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter into an HPLC vial. The sample preparation was performed in duplicate for all the samples considered in this study. The samples were stored at 4°C until analysis was performed [4].

Extraction of PCs from hemp leaves. The extraction of PCs was performed following a previously developed method, slightly modified [21]. A portion of 5 g of leaves was weighed and submitted to extraction with 60 mL of *n*-hexane in an ultrasonic bath for 30 min (Analogic ultrasonic bath Mod. AU–32, Argolab, Italy). The extraction was repeated with the same amount of *n*-hexane. After vacuum filtration, the extracts were pooled and brought to dryness by using a rotary evaporator (Laborota 4000 Heidolph, Schwabach, Germany). The oily residue was dissolved in 15 mL of hot EtOH (75°C) and then placed at − 20°C for 24 h to winterize. The waxy precipitate was centrifugated at 8000 rpm at 2°C for 30 min. After vacuum filtration, the solid residue was washed with 2.5 mL of EtOH and dried overnight in a desiccator. The waxy material was then placed in a closed glass vial with 2.5 mL of EtOH and 50 mg of

KOH, and subjected to a microwave-assisted extraction (MAE) with a microwave apparatus (Biotage Initiator Sixty, Biotage, Sweden) to promote the *trans*-esterification and hydrolysis reactions of long-chain esters and fatty acids. The MAE conditions were set as follows: 80°C for 30 min under medium speed magnetic stirring. The resulting greyish suspension underwent vacuum filtration, washed with 5 mL of H_2O, and finally dried in a desiccator overnight. Finally, 5 mg of the residue was dissolved in 720 µL of $CHCl_3$ and filtered through a 0.45 µm PTFE filter prior to the HPLC-ELSD of PCs. The extraction procedure was performed in duplicate for all the samples considered in this study. Samples were stored at − 20°C until analysis was performed.

Isolation and characterization of squalene from hemp leaves. CBD-type hemp leaves, collected in September 2024, were arranged in a thin layer on stainless-steel trays and dried at room temperature. After 4 days, the dried vegetable material (520 g) was pulverized, extracted with acetone (1:10, w/v, 2 × 12 h) in a vertical stainless-steel percolator at room temperature, filtered in sintered funnel to avoid vegetal material residue, evaporated at reduced pressure (556 mbar, 40°C) to finally obtain a dark green syrup (29 g, 5.7% yield).

Depigmentation occurred on the extracts by solid-phase filtration under vacuum on C_{18} silica gel. For this purpose, the extract was dissolved in EtOH (1:2, w/v) at 40°C and charged on C_{18} (raw extract/stationary phase ratio of 1:3 w/w), protected by a layer of Celite®, packed with EtOH in a sintered funnel (9 × 15 cm) with a side arm for vacuum and eluted with EtOH to obtain the ethanolic fraction (18.6 g) after evaporation at reduced pressure (337 mbar, 40°C).

The ethanolic fraction was vacuum filtered on basic alumina (90 g, PE-EtOAc gradient from 95:5 to 60:40) to obtain four fractions (I-IV). Fraction II (4.8 g) was further purified with flash chromatography (Isolera One with DAD) on silica (50 g, PE-isopropanol gradient from PE 100:0 to 98:2, monitor λ 205 nm) to obtain, after solvent evaporation at reduced pressure (137 mbar, 40°C), 57 mg of a fraction concentrated in squalene as a viscous yellow oil, which was identified according with ^{1}H NMR data previously described in scientific literature [31]. ^{1}H 400 MHz and ^{13}C 100 MHz NMR spectra were recorded with a Bruker 400 spectrometer (Bruker®, Billerica, MA, USA). Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H = 7.25). ^{1}H NMR data are shown in Fig. S1 of the Supporting Information (SI, Fig. 1).

Squalene extraction method from hemp leaves. The extraction procedure of squalene was performed following the method developed and optimized in this study. A weighed amount of sample (1 g) was extracted with 10 mL of EtOAc for 4 h by means of dynamic maceration. The extract was filtered through a paper filter and adjusted to the final volume of 10 mL. Then, the solution was filtered through a 0.45 µm PTFE before the injection into GC-MS and GC-FID instruments. The extraction procedure was performed in duplicate for all the samples considered in this study. Samples were stored at − 20°C until analysis was performed.

Extraction of terpenes from hemp leaves. A portion of 0.5 g of finely ground leaves was treated with 5 mL of EtOAc at room temperature for 15 min on an orbiting shaker. The extract is filtered on a 0.45 µm Nylon syringe filter, dodecane was added to a final concentration of 50 µg/mL as the internal standard and the resulting solution was analyzed [38–40].

Polyphenol profiling and quantification. The qualitative analysis of polyphenols in hemp leaves was performed on a Thermo Scientific (Massachusetts, United States) UHPLC Ultimate 3000 equipped with a vacuum degasser, a binary pump, a thermostatted autosampler, a thermostatted column compartment and a Q-Exactive Orbitrap mass spectrometer with a heated electro-spray ionization (HESI) source (Thermo Scientific, Massachusetts, United States). An Ascentis® Express C_{18} column (150 mm × 3.0 mm I.D., 2.7 µm, Supelco, Bellefonte, PA, USA) was used for the separation of the target compounds. The separation of the compounds was achieved by using a gradient elution with a mobile phase composed of 0.1% HCOOH in H_2O (solvent A) and 0.1% HCOOH in ACN (solvent B). The gradient elution was modified as follows: 0–20 min from 2 to 25% B, 20–30 min from 25 to 40% B, 30–40 min from 40 to 80% B, which was kept for 5 min, 45–55 min from 80 to 90% B, which was kept for 5 min. The post-running time was 10 min for re-equilibration of the system. The flow rate and injection volume were set at 0.3 mL/min and 3 µL, respectively. The column temperature was set at 30°C. MS acquisition was carried out with a heated electro-spray ionization source (HESI) operated in both the positive and in the negative ion mode. For what concerns the MS detector, the source parameters were set as follows: sheath gas (N_2) 37, auxiliary gas (N_2) 28, electrospray voltage 3.4 kV (+) and 2.9 kV (−). The capillary temperature was set at 320°C. The analysis was acquired at a resolving power of 35.000 full width at half maximum (FWHM). The other mass analyzer parameters were set as follows: scan range 100–1000 *m*/z, AGC target 1×10^6 ions in the Orbitrap analyzer, maximum ion injection time (IT) of 243 ms. Data acquisition in data dependent MS/MS (dd–MS/MS) mode was performed at 17.500 resolution, the AGC target was set to 5×10^5 with a maximum IT of 80 ms and an isolation window for the filtration of the precursor ions of 1 *m/z*. The scan range was 200–2000 *m/z.* The fragmentation of precursors ions was performed at 20, 50 and 75 as normalized collision energies (NCE) [16].

The HPLC-UV/Vis analysis for the quantification of polyphenols was performed on an Agilent Technologies (Waldbronn, Germany) modular model 1260 Infinity II Vialsampler system, consisting of a quaternary pump, an autosampler injection and a UV variable wavelength detector under the same chromatographic conditions applied for the UHPLC-HRMS analysis. Chromatograms were recorded using an Agilent OpenLab (3.7 version). Chromatograms were acquired at 210 nm and 342 nm for canniprene and cannflavins, respectively [16]. The standard solutions of CFL-A and CFL-B were prepared by dissolving 1 mg in EtOH to obtain a 1 mg/mL stock solution. The calibration curve of cannflavins was built with five points in the range of 5–100 µg/mL. Canniprene calibration curve covered a range of 1–25 µg/mL. Two injections were performed for each standard solution and sample. The limit of detection (LOD) and the limit of quantification (LOQ) were 1.0 and 3.4 µg/mL for CFL-A and 0.5 and 1.5 µg/mL for CFL-B, and, respectively. For canniprene, the LOD and LOQ were 0.1 and 0.4 µg/mL, respectively.

CB profiling and quantification. The qualitative analysis of CBs was performed by using the same instruments

described in the previous paragraph. An Ascentis® Express C_{18} column (150 mm × 3.0 mm I.D., 2.7 µm, Supelco, Bellefonte, PA, USA) was used for the separation of the target compounds. According to a previously reported method, slightly modified [41], the chromatographic conditions for the qualitative analysis of CBs consisted of a binary gradient elution by using 0.1% HCOOH in H_2O (solvent A) and 0.1% HCOOH in ACN (solvent B) as the mobile phase. The gradient elution program was set up as follows: the initial conditions were 50% B then raised to 67% B in 2 min, held at 67% B for 4 min and then raised to 90% B until 10 min, kept at 90% B until 14 min, decreased to 50% B over the next min, and held at 50% B until 20 min for re-equilibration of the system. A flow rate of 0.3 mL/min was used. The injection volume was 3 µL. The column temperature was set at 30°C. As for MS acquisition, the HESI source was operated both in the positive and the negative ion mode. The MS source parameters were set as follows: sheath gas flow rate (N_2) 37, auxiliary gas flow rate (N_2) 28, capillary temperature: 320°C, electrospray voltage: 3.4 kV (+) and 2.9 kV (−). MS was operated in the full MS mode, followed by data-dependent MS/MS mode. Data acquisition in full MS mode was performed at 70.000 resolution, and the AGC target was set to 1×10^6 with a maximum ion injection time (IT) of 243 ms. The scan range was 100–1000 *m/z* for all acquisition events. Data acquisition in data-dependent MS/MS (dd–MS/MS) mode was performed at 17.500 resolution; the automatic gain control (AGC) target was set to 5×10^5 with a maximum IT of 120 ms and an isolation window of 3 *m/z*. The scan range was 200–2000 *m/z*. To study the response of the major product ions of selected CBs with energy, the samples were analyzed in the range of 20, 30, 50 NCE levels [41].

To improve the identification of CBDA esters, the aforementioned method was optimized for MS parameters. Conversely to the previous approach, the acquisition was operated exclusively in positive ion mode, and the scan range explored was 300–1000 *m/z*.

The quantitative analysis of CBs was performed with the same HPLC-UV/Vis equipment as described in the previous paragraph. The mobile phase was composed of a 2 mM ammonium formate solution and 0.1% HCOOH in H_2O (solvent A) and 0.1% HCOOH in ACN (solvent B). According to a previous work [29], the gradient was set as follows: 0–20 min from 70% to 90% B, which was held for 5 min with a 10 min post-running time. Flow rate and injection volume were set at 0.2 mL/min and 3 µL, respectively. Chromatograms were recorded at the wavelength of 210 and 220 nm for the detection of neutral cannabinoids and cannabinoid acids, respectively [29]. Standard solutions for acidic CBs (CBDVA, CBDA, CBGA, THCVA, CBNA, THCA and CBCA) and neutral CBs (CBDV, CBG, CBD, CBN, THCV and CBC) were diluted with EtOH to reach a stock solution of 100 µg/mL. A five-point calibration curve was generated covering the concentration range of 5–100 µg/mL, while the Δ^9-THC calibration curve was in the concentration range of 2.5–50 µg/mL. The stock standard solutions for CBDBA and CBDB were prepared as follows: 1 mg of the compound was dissolved in 1 mL of MeOH reaching a 1 mg/mL solution, then diluted to generate a five-points calibration curve in the concentration range of 5–100 µg/mL. Three

injections were performed for each standard solution, and two injections for each sample. The LOD values obtained for acidic cannabinoids ranged from 0.3 to 1.5 µg/mL, while those for neutral cannabinoids ranged from 0.2 to 0.8 µg/mL. The LOQ values were in the range of 1.1–2.6 µg/mL for acidic cannabinoids and 0.6–2.6 µg/mL for neutral cannabinoids.

PC profiling and quantification. The analysis of PCs was performed on an Agilent Technologies (Waldbronn, Germany) modular model 1260 Infinity II system, consisting of a quaternary pump, a thermostatted column compartment, and an evaporative light scattering detector (ELSD). The separation occurred on an Atlantis™ dC_{18} column (150 ×3.0 mm, 3 µm, Waters, Milford, MA, USA). A gradient elution with a mobile phase composed of ACN (solvent A) and MTBE-MeOH 90:10 (*v/v*) (solvent B) was used for the separation, which was set as follows: 0–1 min isocratic elution at 20% B, 1–16 min linear gradient from 20% to 45% B, which was held constant for 4 min, with a post-running time of 5 min. The flow rate was set at 1.5 mL/min, and the injection volume was 10 µL. The ELSD evaporator temperature was set at 35°C, while the nebulizer temperature was 30°C. Nitrogen flow rate was set at 1.50 SLM [21]. An accurate amount of $C_{30}OH$ reference standard (10 mg) was weighed and dissolved in $CHCl_3$ in a 5 mL volumetric flask as a stock solution. A four-point calibration curve was built covering the ranges of 25–100 µg/mL. Three injections were performed for each standard solution, and two injections for each sample. The LOD and LOQ values for $C_{30}OH$ were 35.0 and 116.6 µg/mL, respectively.

Squalene identification and quantification. Squalene was analyzed by means of GC-MS and GC-FID techniques. GC-MS analyses were carried out on a 7890 B GC System (Agilent Technologies, Waldbronn, Germany), coupled with a 5975C network mass spectrometer (Agilent Technologies, Germany). Compounds were separated on an Agilent HP-5MS capillary column (30 m × 0.25 mm I.D., 0.25 µm film thickness, Agilent Technologies). The oven temperature was initially set at 150°C, then increased to 320°C at a rate of 4°C/min, this final temperature being kept for 15 min. The injection volume was 1 µL, with a 1:20 split ratio. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The injector and the transfer line temperature were set at 330°C. Electron ionization (EI) at 70 eV was used to perform MS detection, operating in the full-scan acquisition mode in the *m/z* range 50–600. Peak identification was performed through a search of mass spectra in the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) mass-spectral database (version 2.0d, 2005) [22].

GC-FID analyses were performed by using a Shimadzu GC–2010 system (Shimadzu Corporation, Kyoto, Japan), equipped with a split/splitless injector and a flame ionization detector (FID). The column used was a HP–5MS capillary column (30 m × 0.25 mm I.D., 0.25 m film thickness, Agilent Technologies). The GC-FID conditions were the same as those described in the literature [22], with slight changes: the initial column temperature was set at 150°C, programmed to increase at a rate of 4°C/min until 300°C, and then held constant for 15 min. The injector and detector temperatures were 280 and 300°C, respectively. The injection volume was 1 µL, with a split ratio of 1:20. The injection was performed by using the three-layer sandwich mode, with 0.5 µL of EtOAc, another 0.5 µL air gap and 1.0 µL of sample. Helium (He) was used as the carrier gas at a flow rate of 1 mL/min [22]. A squalene stock standard solution of 1

mg/mL in EtOAc was prepared, obtaining a five-point calibration curve in the concentration range of 1–25 µg/mL. Two injections were performed for each standard solution and sample. The LOD and LOQ values of squalene were 2.3×10^{-3} µg/mL and 7.7×10^{-3} µg/mL, respectively.

Terpene profiling and quantification. The qualitative analysis of terpenes was performed by GC-MS analyses with an Agilent Technologies 7890A single quadrupole GC/MS system (Agilent 5975C mass spectrometer – Agilent Technologies, Santa Clara, California, USA).

Chromatographic separation was performed on a HP-5MS capillary column (30 m length × 0.25 mm ID, 0.25 µm film thickness, Restek, Milan, Italy) with helium as the carrier gas at a constant flow-rate of 1.0 mL/min. An injection volume of 1 µL was employed. The injector temperature was set at 250°C and operated in the splitless mode. The oven temperature was programmed from 60 to 115°C at the rate of 3°C/min, followed by a ramp to 250°C at the rate of 10°C/min (toral run time: 32 min). Data acquisition started 5 min after the injection. Mass transfer line temperature was set at 300°C. All mass spectra were acquired with an electron ionization system (EI, Electron Impact mode) with ionization energy of 70 eV and source temperature of 250°C, with spectral acquisition in Full Scan mode, positive polarity, in the *m/z* range 50–600. Data were analyzed by MSD 5975 VL data analysis software (Agilent Technology).

Diluted standards were prepared in EtOH to obtain five different concentrations (from 2 to 100 µg/mL), and dodecane (50.0 µg/mL) was added as the internal standard [38–40]. The LOD and LOQ values were 0.07 µg/g and 0.2 µg/g for each terpene.

Declarations

Competing interests

The authors declare no competing interests.

Supplementary information

Additional information is available at the link below.

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Author Contribution

Conceptualization: Daniele Merli, Gianni Sacchetti, Federica Pollastro, Federica Pellati. Methodology: Matilde Marani, Aurora Camola, Caterina Fantino, Virginia Brighenti, Massimo Tacchini, Maria Eleonora Foletti. Investigation and formal analysis: Matilde Marani, Aurora Camola, Caterina Fantino, Virginia Brighenti, Massimo Tacchini, Maria Eleonora Foletti. Writing original draft preparation: Matilde Marani, Aurora Camola, Daniele Merli. Writing review and editing: Massimo Tacchini, Daniele Merli, Gianni Sacchetti, Federica Pollastro, Federica Pellati. Funding acquisition: Daniele Merli, Gianni Sacchetti, Federica Pollastro, Federica Pellati. Resources: Daniele Merli, Gianni Sacchetti, Federica Pollastro, Federica Pellati. Supervision: Federica Pollastro, Federica Pellati.

Data Availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Figures

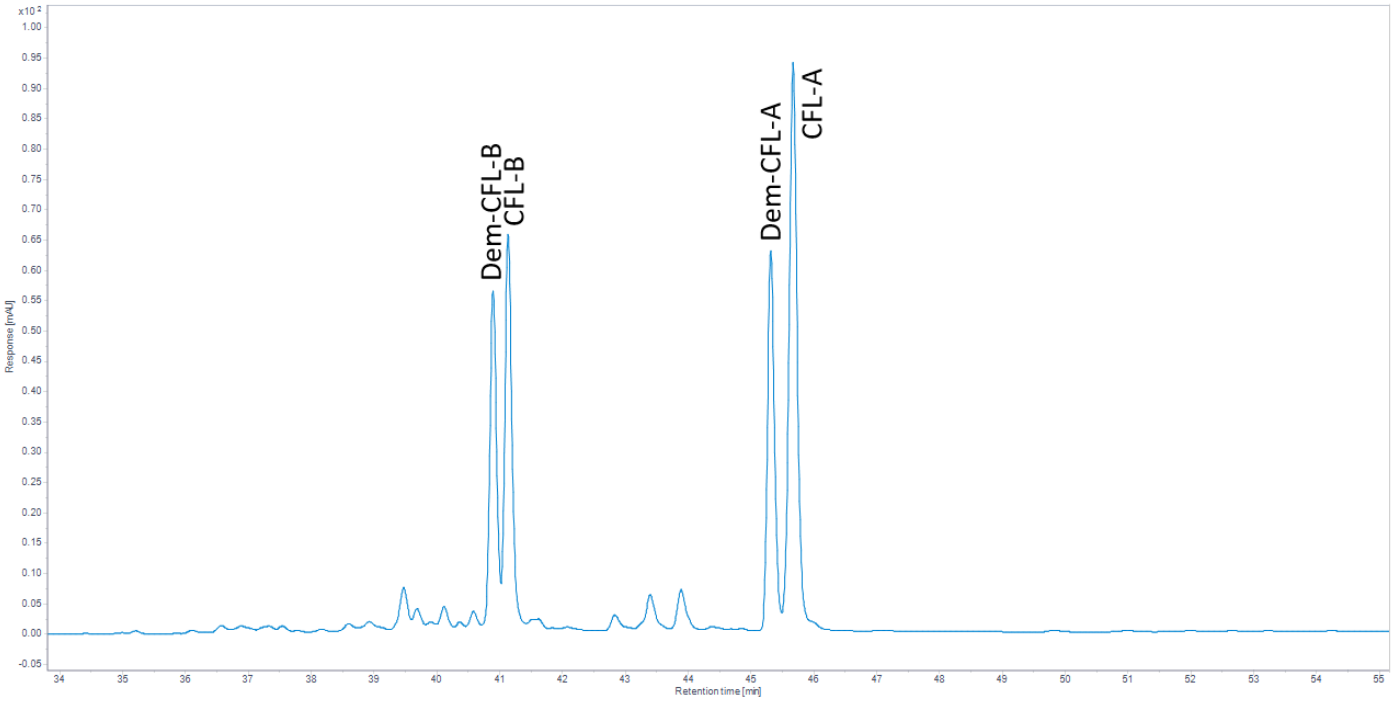

Figure 1

HPLC-UV/Vis representative chromatogram of PFs in a leaf extract from the CBD-type variety (sample 1), acquired at 342 nm.

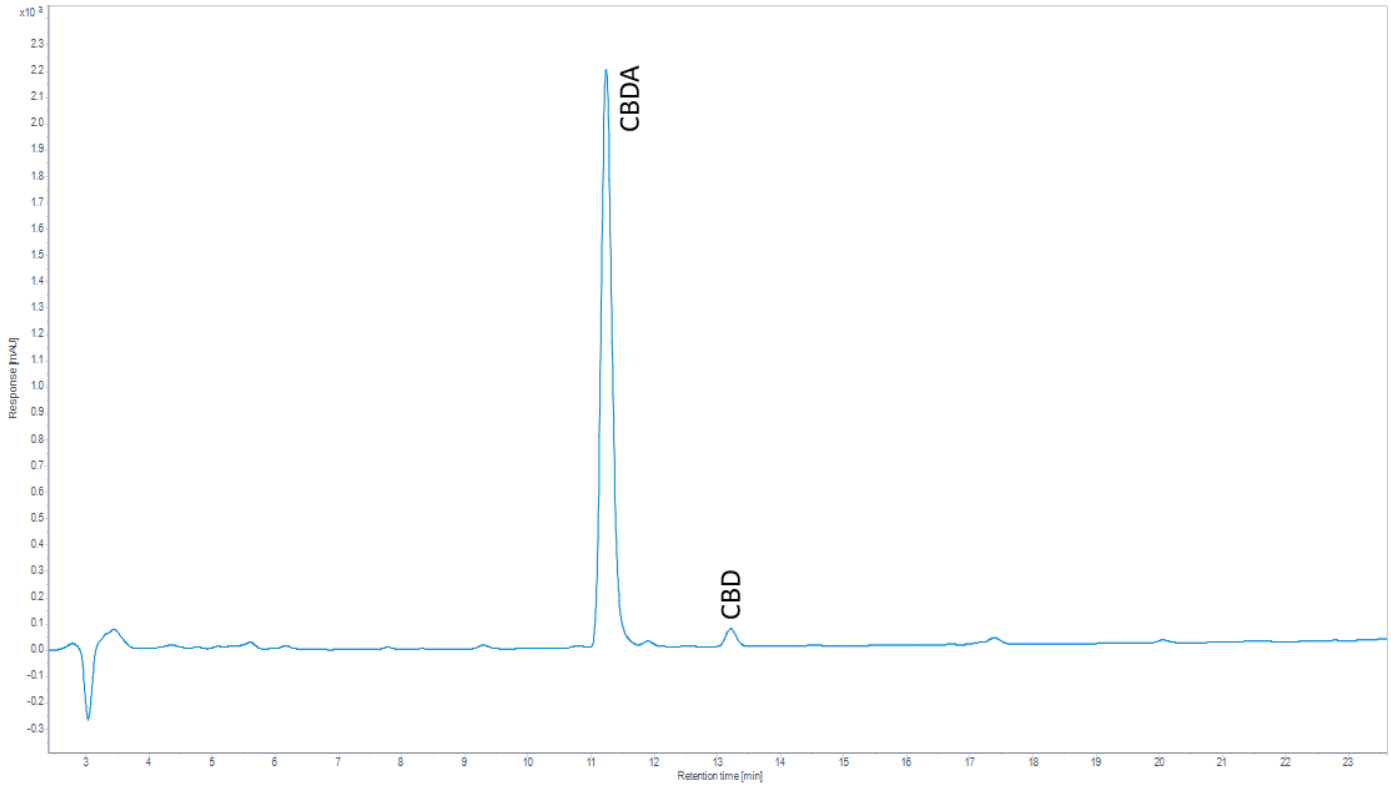

Figure 2

HPLC-UV/Vis representative chromatogram of CBs in a hemp leaf extract from the CBD-type variety (sample 1), acquired at 220 nm.

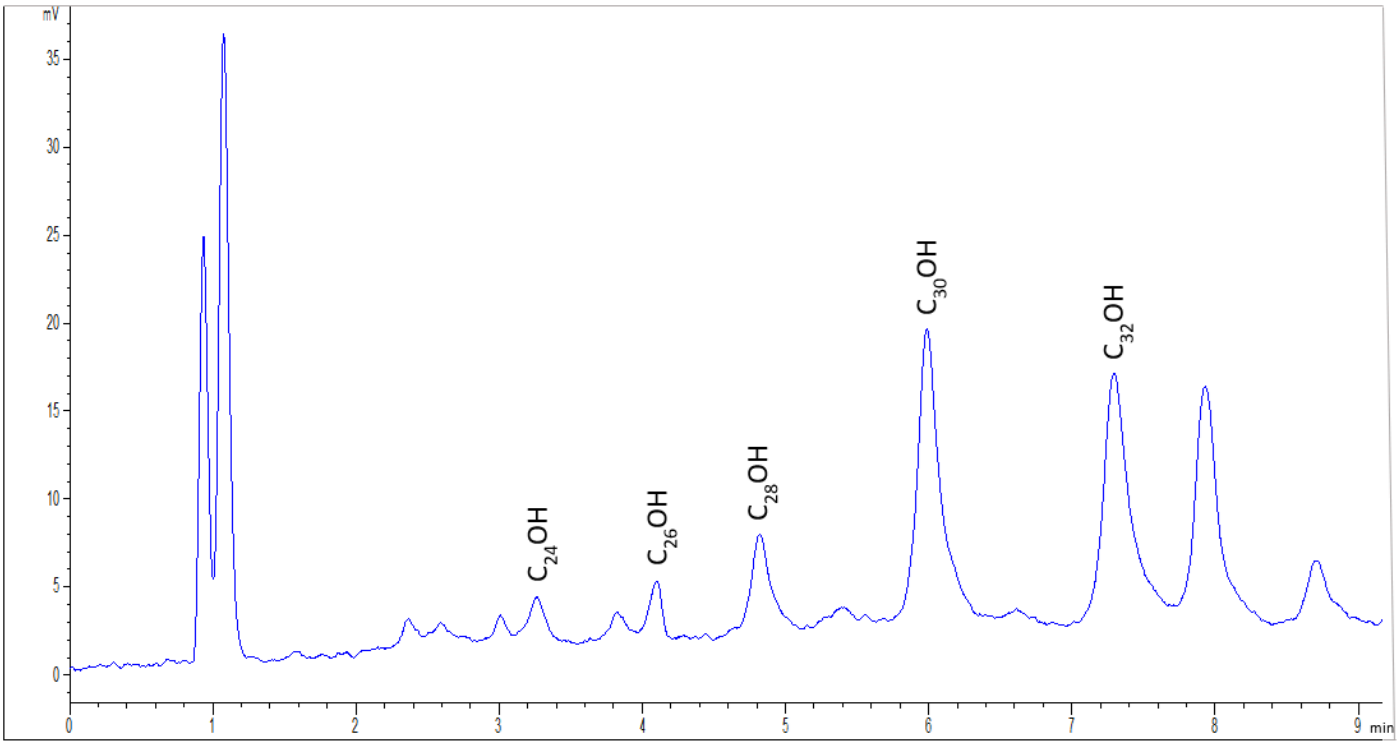

Figure 3

HPLC-ELSD representative chromatogram of PCs in a hemp leaf extract from the CBD-type variety (sample 1).

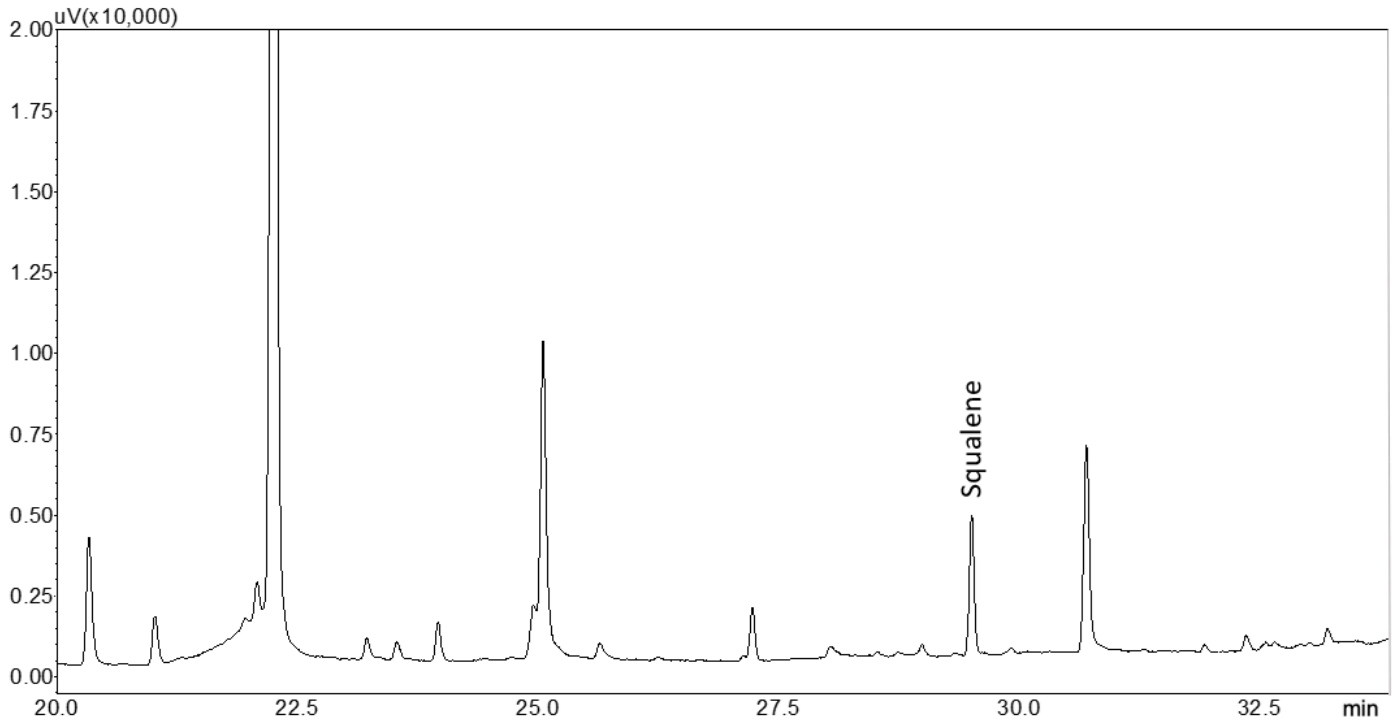

Figure 4

GC-FID representative chromatogram of squalene in a leaf extract from the CBC-type variety (sample 3).

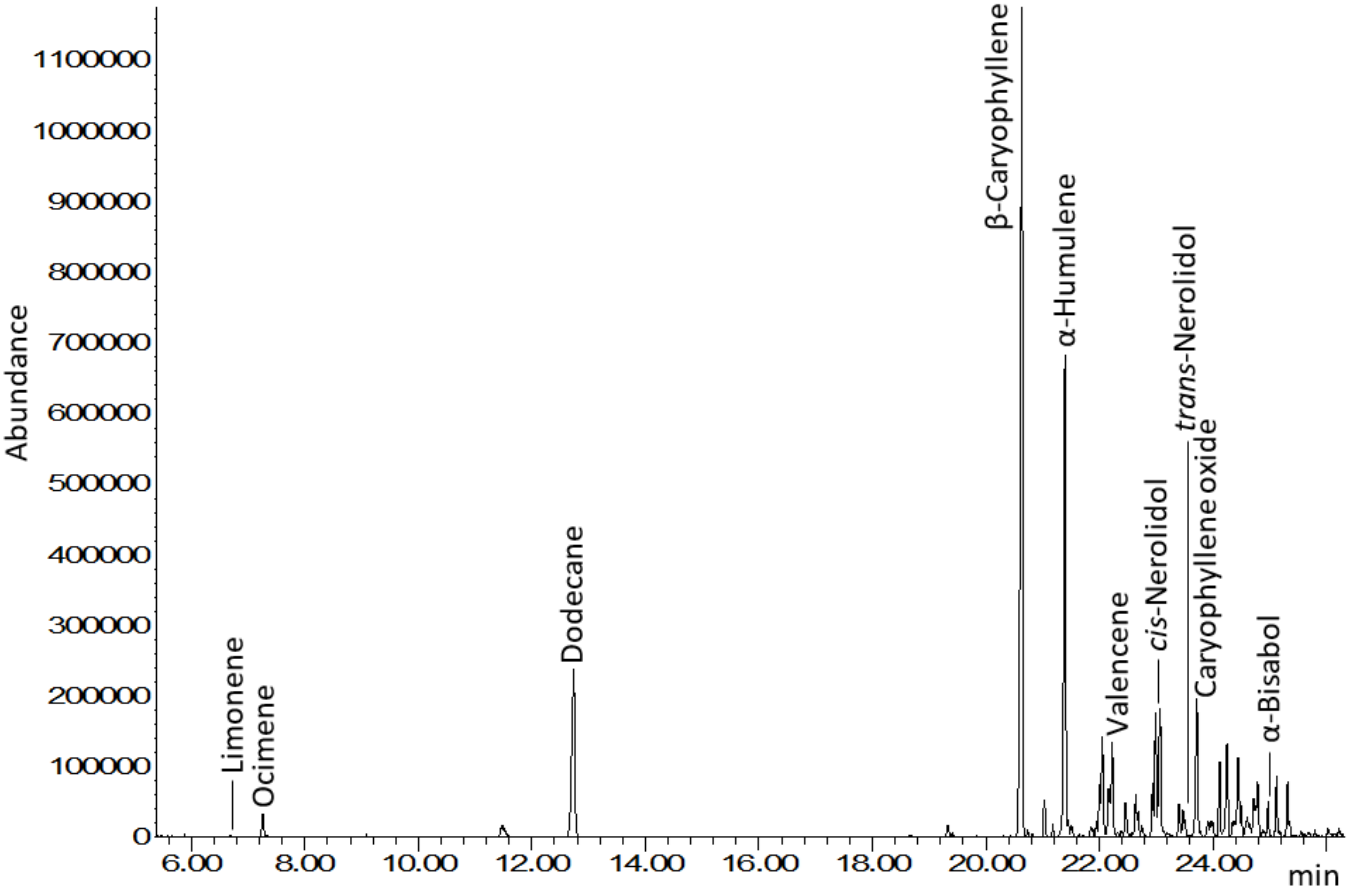

Figure 5

GC-MS representative chromatogram (TIC) of terpenes in a leaf extract from the CBC-type variety (sample 3).

Supplementary Files

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