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應用手冊

Strategies for Targeted and Non-Targeted Screening and Differentiation of Cannabis Cultivars Using UPLC and APGC with Quadrupole Time-of-Flight Mass Spectrometry

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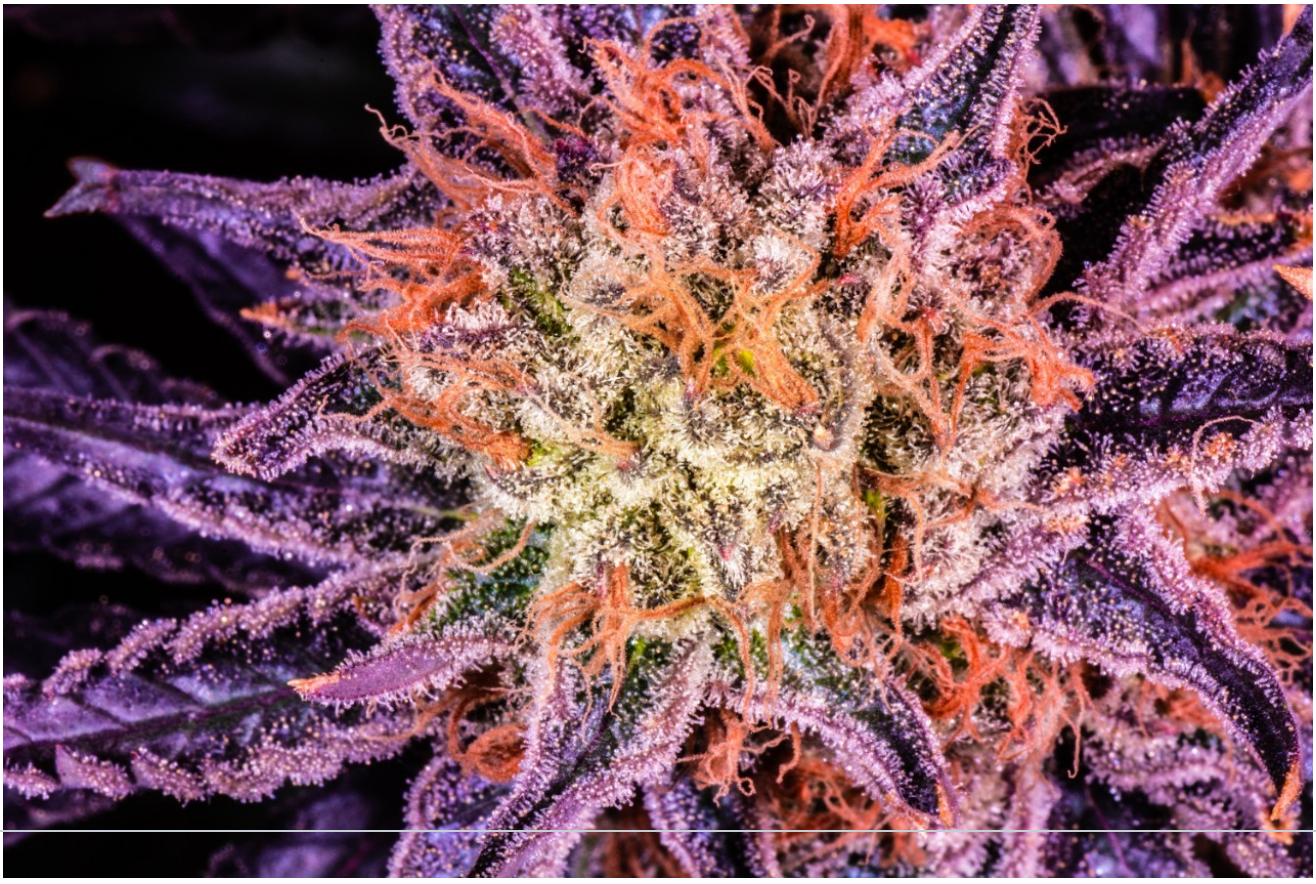


Abstract

This application note demonstrates a workflow for chemical profiling of cannabis cultivars.

Benefits

- Profiling of cannabis using LC and GC separations and a single informatics workflow and HRMS system
- Enhanced confidence in compound identifications using simultaneous collection of accurate mass precursor and product ion data combined with compound libraries
- Progenesis QI facilitates interrogation of the complex and variable chemistry of cannabis

Introduction

The use of cannabis for both medicinal and adult use purposes is increasing and gaining acceptance[1] with many countries implementing official programs to provide access to safe and high-quality cannabis products. Cannabis has shown promising therapeutic potential in the treatment of a diverse array of medical conditions including chronic pain and seizure disorders.[2-4] Cannabis varieties can vary immensely in their chemical composition[4-8] and understanding the chemical variation of the cultivars and its relevance to therapeutic effects and user experience is important. Comprehensive chemical profiling can aid in establishing identities using quantifiable markers that allow for the differentiation of various plant chemistries with the goal of correlating the chemical profile with the pharmacological effects.[9-13] Attempts have been made to classify cannabis cultivars based on chemical profiles including both phytocannabinoid and terpenoid profiles.[10-21] The chemical profile (chemotype) data is used in combination with genotyping for more complete information.[20,21] These specific profiles were monitored, as they are considered to be the main active components in the plant and have been reported to exert a synergistic relationship that greatly influences the beneficial effects of cannabis.[15]

In this study, a workflow for chemical profiling of cannabis cultivars will be demonstrated. Cannabis flower samples from 18 cultivars including hemp were extracted and analyzed (n=5) using UltraPerformance Liquid Chromatography (UPLC) and atmospheric pressure gas chromatography (APGC) with a high-resolution time-of-flight mass spectrometer (Tof-MS). The combination of these technologies provides greater analytical coverage, which can help interrogate the composition of complex samples like cannabis and hemp. In-house cannabinoid and terpene reference databases were used to assign identities to the compounds detected. These

databases were generated *in-silico* and from experimental data. Mass spectral information is predicted from chemical structures of known cannabinoids and terpenes supported by experimental data generated through the analysis of available authentic reference standards, where available.

The databases consisted of accurate masses of molecular ions and fragment ions, isotope patterns and, in cases where reference standards are available, additional chromatographic properties such as retention times, which were used to identify components. Multivariate analyses (MVA) such as principal component analysis (PCA) were used to identify the differences in chemical composition between various cannabis cultivars.

The concentrations of chemical constituents produced by the plant are dependent on many factors including environmental conditions such as light, soil, plant age, growth conditions, harvest time, etc.[14,22] It should be noted that the study did not allow for the control of environmental variables that are known to exert a significant influence on the plant chemistry.

Experimental

Conditions

Cannabinoid Sample Preparation

Cannabinoid authentic standard solutions for the 16 compounds (Table 1) were combined to make a stock solution in acetonitrile. Cannabis flowers were collected from 18 different cultivars including hemp and homogenized separately. Homogenized plant material (0.1 g) was weighed into 50 mL centrifuge tubes. A 5 mL volume of acetonitrile was added, and the samples were processed using a Geno Grinder for 2 minutes (1000 rpm). The samples were then centrifuged at 5000 rpm for 5 minutes. The supernatant was removed and further diluted 1:10 in preparation for analysis by UPLC-Tof-MS.

Name	MWT	Chemical formula	CAS
Δ^9-Tetrahydrocannabinol (D9-THC)	314.2246	$C_{21}H_{30}O_2$	1972-08-03
Δ^8-Tetrahydrocannabinol (D8-THC)	314.2246	$C_{21}H_{30}O_2$	5957-75-5
Δ^9-Tetrahydrocannabinolic acid A (THCA)	358.2144	$C_{22}H_{30}O_4$	23978-85-0
Cannabidiol (CBD)	314.2246	$C_{21}H_{30}O_2$	13956-29-1
Cannabidiol acid (CBDA)	358.2144	$C_{22}H_{30}O_4$	1244-58-2
Cannabigerol (CBG)	316.2402	$C_{21}H_{32}O_2$	25654-31-3
Cannabigerolic acid (CBGA)	360.2301	$C_{22}H_{32}O_4$	25555-57-1
Cannabichromene (CBC)	314.2246	$C_{21}H_{30}O_2$	20675-51-8
Cannabichromenic acid (CBCA)	358.2144	$C_{22}H_{30}O_4$	185505-15-1
Tetrahydrocannabivarin (THCV)	286.1933	$C_{19}H_{26}O_2$	31262-37-0
Tetrahydrocannabivarinic acid (THCVA)	330.1831	$C_{20}H_{26}O_4$	39986-26-0
Cannabidivarin (CBDV)	286.1933	$C_{19}H_{26}O_2$	24274-48-4
Cannabidivarin acid or cannabidivarinic acid (CBDVA)	330.1831	$C_{20}H_{26}O_4$	31932-13-5
Cannabicyclol (CBL)	314.2246	$C_{21}H_{30}O_2$	21366-63-2
Cannabicyclolic acid (CBLA)	358.2144	$C_{22}H_{30}O_4$	40524-99-0
Cannabinol (CBN)	310.1933	$C_{21}H_{26}O_2$	521-35-7

Table 1. Names, molecular weight, elemental composition, accurate mass, and CAS numbers of the target cannabinoids analyzed in the study.

Terpenes Sample Preparation

A standard mix containing 23 terpenes in isopropanol (Table 2) was used to make stock solutions for the terpene analysis. 0.1 g of homogenized plant material was weighed into a 20 mL scintillation vial. Five milliliters of ethyl acetate were added to the vial. After sonication for 15 mins approximately 4 mL of the resultant extract was transferred to a 4 mL amber vial. Samples were centrifuged and a portion was transferred to 2 mL autosampler vials for analysis by GC-MS. This sample preparation procedure was adapted from recent work that included investigation of various extraction solvents and included validation of the method using GC-MS.[23]

Name	MWT	Chemical formula	CAS
α-Pinene	136.1252	$C_{10}H_{16}$	80-56-8
Camphene	136.1252	$C_{10}H_{16}$	79-92-5
(-)-β-Pinene	136.1252	$C_{10}H_{16}$	18172-67-3
β-Myrcene	136.1252	$C_{10}H_{16}$	123-35-3
δ-3-Carene	136.1252	$C_{10}H_{16}$	13466-78-9
α-Terpinene	136.1252	$C_{10}H_{16}$	99-86-5
π-Cymene	134.1096	$C_{10}H_{14}$	99-87-6
δ-Limonene	136.1252	$C_{10}H_{16}$	5989-27-5
Ocimene	136.1252	$C_{10}H_{16}$	13877-91-3
γ-Terpinene	136.1252	$C_{10}H_{16}$	99-85-4
Terpinolene	136.1252	$C_{10}H_{16}$	586-62-9
Linalool	154.1358	$C_{10}H_{18}O$	78-70-6
(-)-Isopulegol	154.1358	$C_{10}H_{18}O$	89-79-2
Geraniol	154.1358	$C_{10}H_{18}O$	106-24-1
β-Caryophyllene	204.1878	$C_{15}H_{24}$	87-44-5
α-Humulene	204.1878	$C_{15}H_{24}$	6753-98-6
Nerolidol	223.2062	$C_{15}H_{26}O$	7212-44-4
(-)-Guaiol	223.2062	$C_{15}H_{26}O$	489-86-1
(-)-α-Bisabolol	223.2062	$C_{15}H_{26}O$	23089-26-1
(-)-Caryophyllene oxide	221.1905	$C_{15}H_{24}O$	1139-30-6
Eucalyptol	154.1358	$C_{10}H_{18}O$	470-82-6

Table 2. Names, CAS numbers, and synonyms of the target cannabinoids and terpenes analyzed in the study.

Instrumentation and Software

LC separations were performed on the ACQUITY UPLC I-Class System and the Xevo G2-XS QTof Mass Spectrometer. MassLynx Software was used for data acquisition. Progenesis QI and EZinfo were used for data processing and MVA.[24]

UPLC Method Conditions

Column:	ACQUITY UPLC CSH Phenyl-Hexyl* 2.1 × 100 mm, 1.7 µm
Solvent A:	Water with 0.1% formic acid
Solvent B:	Acetonitrile with 0.1% formic acid
Flow rate:	0.600 mL/min
Column temp.:	30 °C
Injection volume:	1 µL

Gradient

Time (min)	%A	%B	Curve
0.0	45%	55%	–
6.0	40%	60%	6
9.0	20%	80%	6
10.0	1%	99%	6
13.0	1%	99%	6
13.1	45%	55%	1

*Following data acquisition and further evaluation of chromatographic properties, the separation achieved using a CORTECS C_{18} (p/n: 186007096) was found to provide superior resolution of the authentic cannabinoid standards used in the study.[31]

Xevo G2-XS QTof Conditions

Acquisition mode:	MS^E sensitivity mode
Start and end mass:	100–1200 Da
Ionization mode:	ESI+
Capillary voltage:	3.5 kV
Cone voltage:	25 V
Collision energy ramp:	15–35 eV

Desolvation temp.:	400 °C
Source temp.:	100 °C
Desolvation gas flow:	800 (L/Hr)
Cone gas:	50 (L/Hr)

APGC Method Conditions

GC:	Agilent 7890B with 7693A autosampler
Column:	Restek Rxi-5MS, 20 m × 0.18 mm I.D. × 0.18 µm film
Carrier gas:	Helium at 0.4 mL/min
Injection:	1 µL split 20:1 at 275 °C using 4 mm I.D. straight inlet liner with wool
Solvent delay:	4.0 min

GC Oven Program

Rate (°C/min)	Temp. (°C)	Hold (min)
–	40	3.0
12	180	0.0
50	325	6.0
Total Run Time = 23.57 min		

Xevo G2-XS QTof Conditions

Acquisition mode:	MS^E sensitivity mode
Mass range:	40–500 Da
Ionization:	APGC+ protonation mode using water
Corona current:	2.0 µA
Cone voltage:	20 V
Cone gas:	100 L/Hr Nitrogen
Makeup gas:	400 L/Hr Nitrogen
Auxiliary gas:	150 L/Hr Nitrogen
Transfer line:	300 °C

Source temp.:	150 °C
Collision energy ramp:	10–40 eV

Library Generation

A data independent acquisition mode, known as MS^E, was used to collect accurate mass measurements from precursor and product ions in a single injection. The incidence of false positives is significantly reduced when using multiple attributes to search entries in a scientific library or database, greatly increasing confidence in the results. The processed data files from the analysis of available authentic standards, in combination with the relevant structural .mol files, were used to create a custom database of compounds of interest. The retention time and accurate mass information can be harvested from the Progenesis QI report (Figure 1). Additional structures of compounds, for which no reference standards were available, were added to the library to bring the total number of cannabinoids and related compounds to over 120. There are many isomers in the cannabinoid and terpene compound classes, in addition, structural features are often similar, therefore databases containing specific information such as fragmentation and chromatographic retention times will aid in both targeted and untargeted studies. Additional databases can be accessed through ChemSpider (www.chemspider.com < http://www.chemspider.com/> The Royal Society of Chemistry).

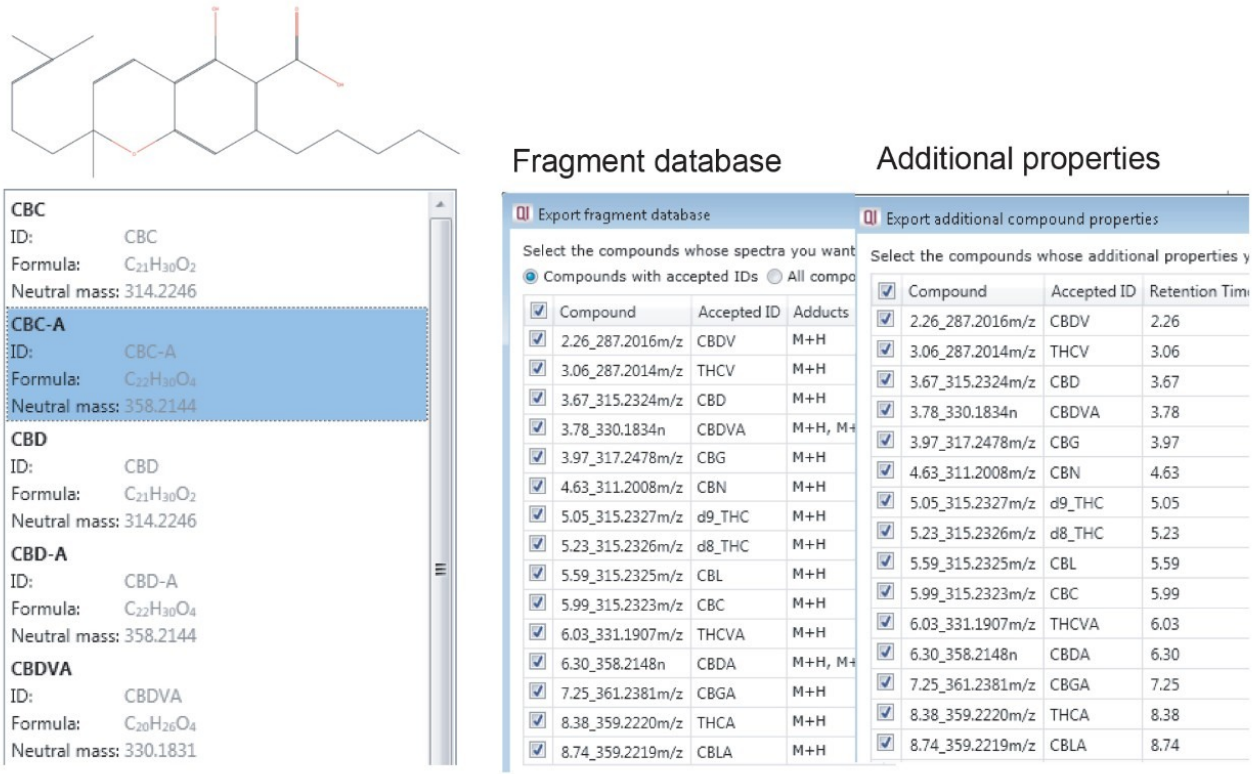


Figure 1. An example of the database entry for CBCA as well as fragment and additional compound properties databases.

Results and Discussion

UPLC-Tof/MS Analysis of Cannabinoids

The authentic standards of 16 cannabinoids were used as the initial target components. Using the chromatographic separation shown in Figure 2 it is possible to identify each cannabinoid in the mix.

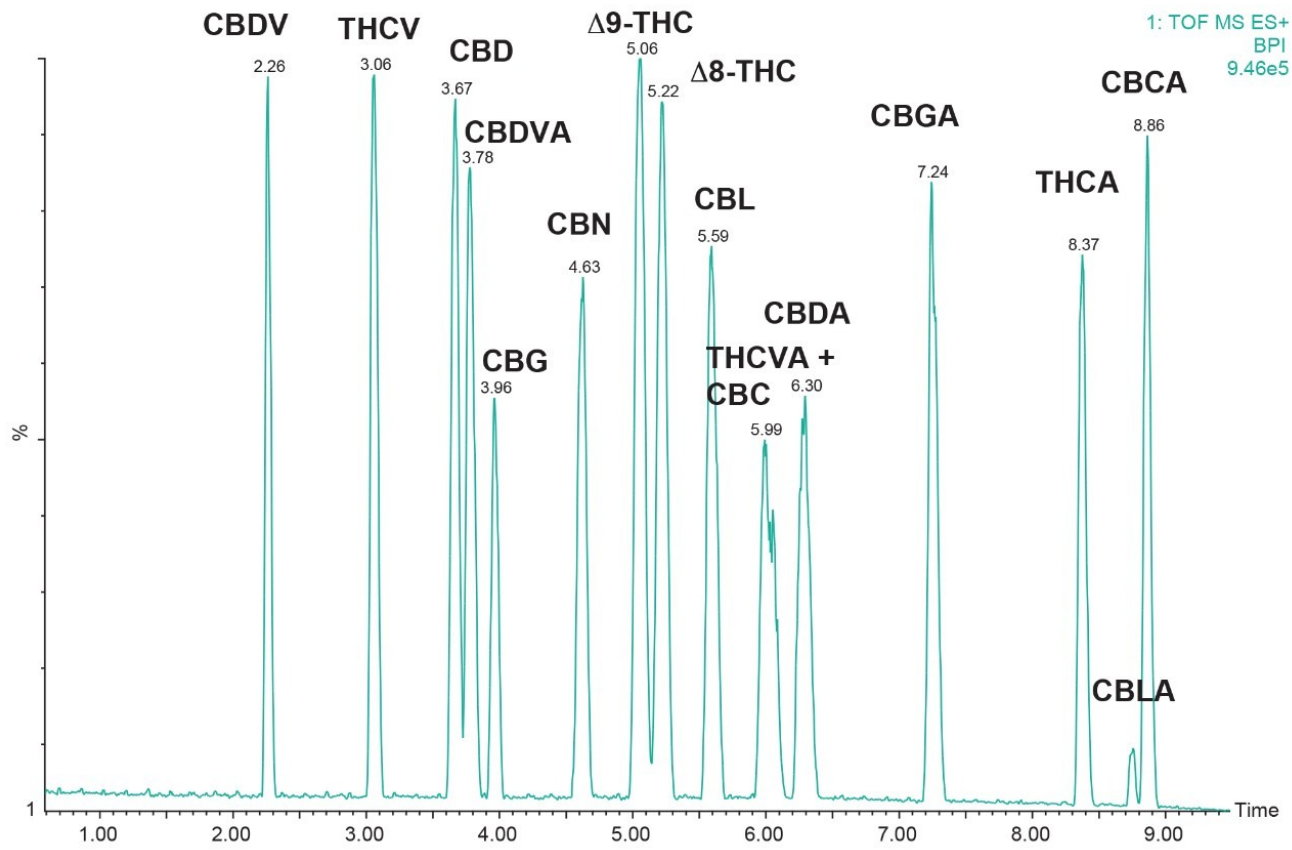


Figure 2. Base peak intensity chromatogram of authentic standard mix of 16 cannabinoids analyzed in the study (1 µL injection).

Differentiation of Cannabis Cultivars

Principal component analysis (PCA) was used to give an overview of the information from the terpene and cannabinoid profiles and to summarize the patterns observed.[24] The PCA plot for the UPLC-Tof-MS analysis of the cannabis cultivars including hemp is shown in Figure 3.

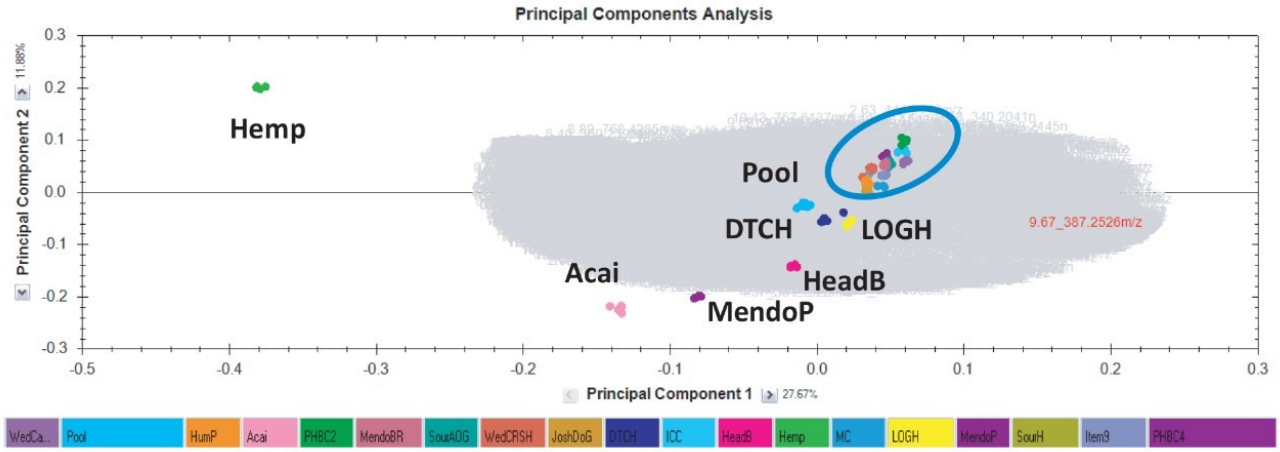


Figure 3. PCA plot for the UPLC-MS analysis of cannabis cultivars including hemp. Each cultivar is represented by a different color.

A cluster of cultivars (highlighted by the blue ellipse) in Figure 3, was observed in the PCA data. Several other variants were segregated indicating that they are chemically distinct. The main distinction observed is between the hemp (higher CBD and low Δ^9-THC variant) on the left of the plot and the high Δ^9-THC cultivars on the right (higher levels of Δ^9-THC). Removal of the hemp group results in the pattern shown in Figure 4 where each drug-type cultivar is clearly differentiated. The Mendo Purps, HumP, and Acai cultivars are the most distinct.

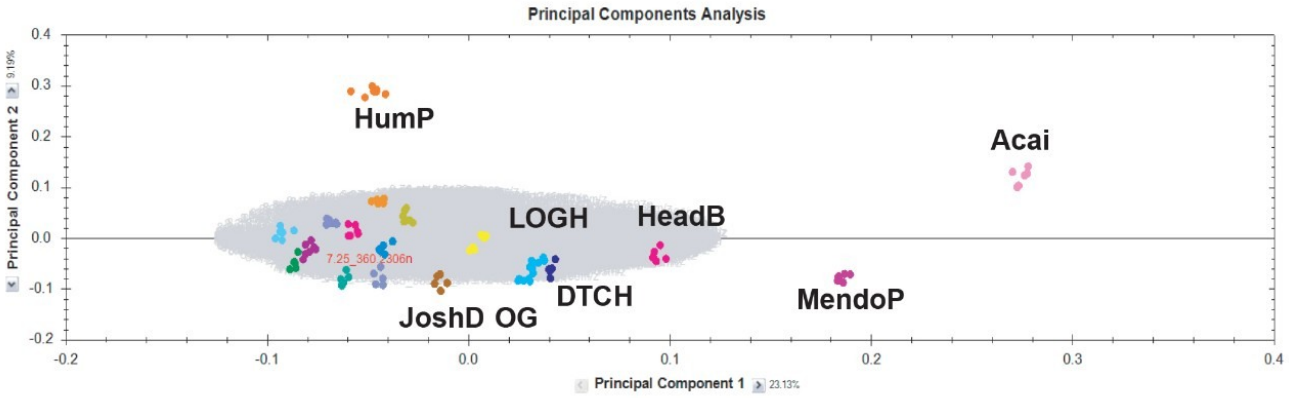
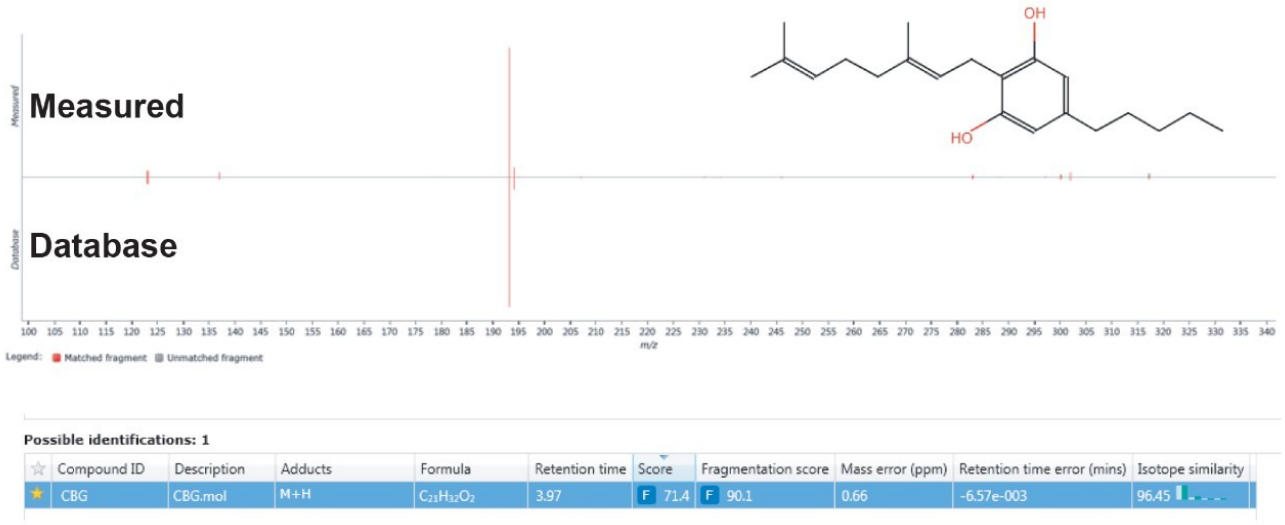


Figure 4. PCA plot for the UPLC-MS analysis of cannabis cultivars excluding hemp.

Identification and Abundance Profile Of CBG

CBG is a non-psychotropic cannabinoid and has been attracting pharmacological interest due to its potential therapeutic properties.[25,26] CBG has been identified using the structure, additional properties, and fragment database. The elemental composition of $C_{21}H_{32}O_2$ is proposed, $[M+H]^+$ 317.2477. The mass error, retention time error and isotope similarity are also favorable increasing confidence in the identification (Figure 5).



Possible identifications: 1

Compound ID	Description	Adducts	Formula	Retention time	Score	Fragmentation score	Mass error (ppm)	Retention time error (mins)	Isotope similarity
CBG	CBG.mol	M+H	$C_{21}H_{32}O_2$	3.97	F 71.4	F 90.1	0.66	-6.57e-003	96.45

Figure 5. CBG identified using the structure, fragmentation, and additional properties databases.

The abundance profile plot for CBG illustrates the similarity or differences in the relative behavior of this cannabinoid across the samples (Figure 6).

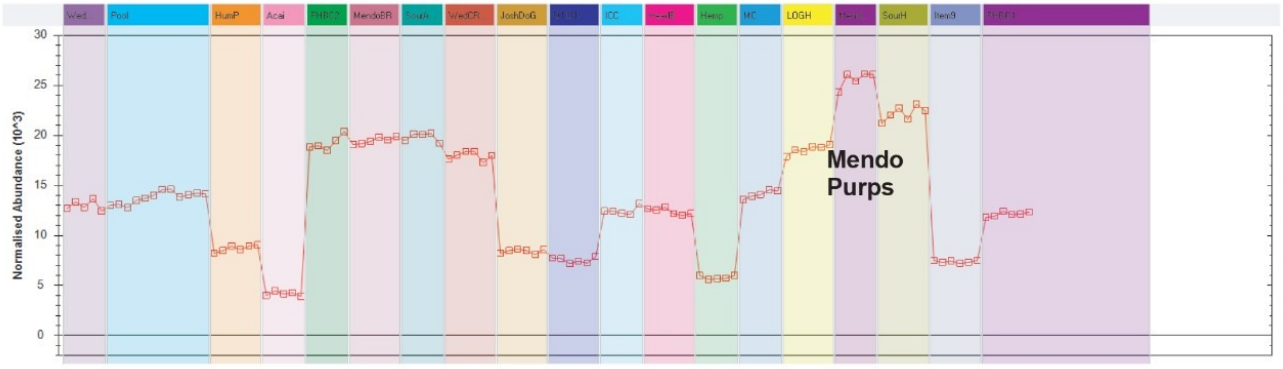


Figure 6. Abundance profile plot showing the difference in the relative behavior of CBG across the cultivars.

In Compound Review, information about the features (*m/z*-retention time pairs) and identified components is tabulated. The information on the varieties with the highest and lowest means (means of relative signal intensities), max fold change as well as other parameters that summarize the variation in the observations is shown (Figure 7). ANOVA p (false positive rate, FPR) and q (false discovery rate, FDR) tests are used to measure the significance of the difference for each feature in the data matrix.[27] The highest and lowest mean for CBG were found in the Mendo Purps and Acai cultivars, respectively, with a fold change of 6.15.

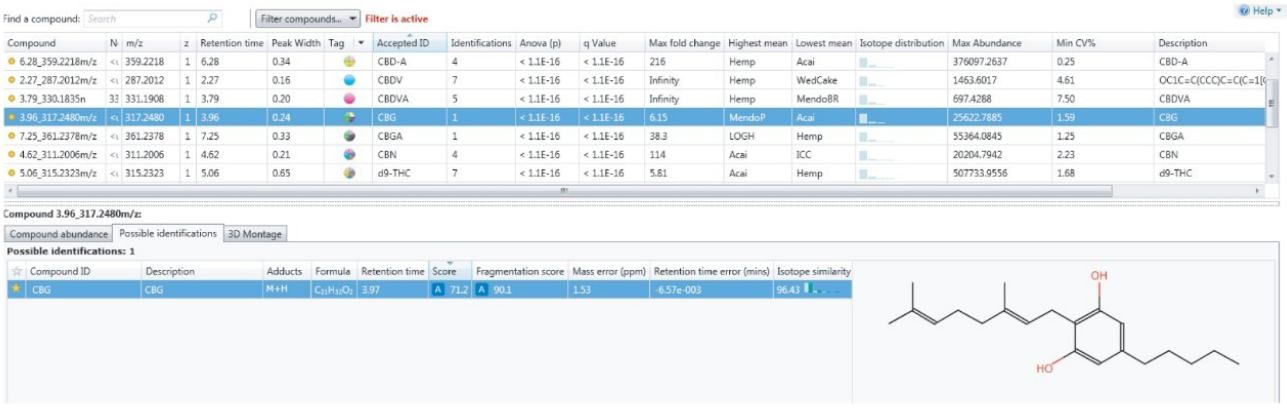


Figure 7. Review compounds provides a summary of data for the identified cannabinoids and other features.

For extended MVA, Progenesis QI can export the data to EZinfo, where the loadings biplot for the detected cannabinoids are observed (Figure 8). The plot can reveal trends and similarities among the observations. The markers for Δ^9-THC and THCV are oriented towards the top left quadrant. In potency experiments performed using UPLC/UV (data not shown), the cultivars in the top left quadrant were predominantly found to have higher Δ^9-THC levels ranging from 0.91–2.97% weight while the majority of cultivars tending towards the lower left quadrant were principally found to test higher for THCA. In addition, the cannabinoids markers, THCVA, CBCA, CBGA, and CBG are shown in this quadrant indicating that these cultivars show greater expression for these cannabinoids. Clustered towards the hemp group on the right side of the plot are the cannabinoids CBD, CBDA, and CBDV. CBN, a metabolite of Δ^9-THC, was observed in the upper quadrant indicating the possibility that the cultivars in this section of the plot were either aged or were subjected to storage conditions that led to its formation.[13]

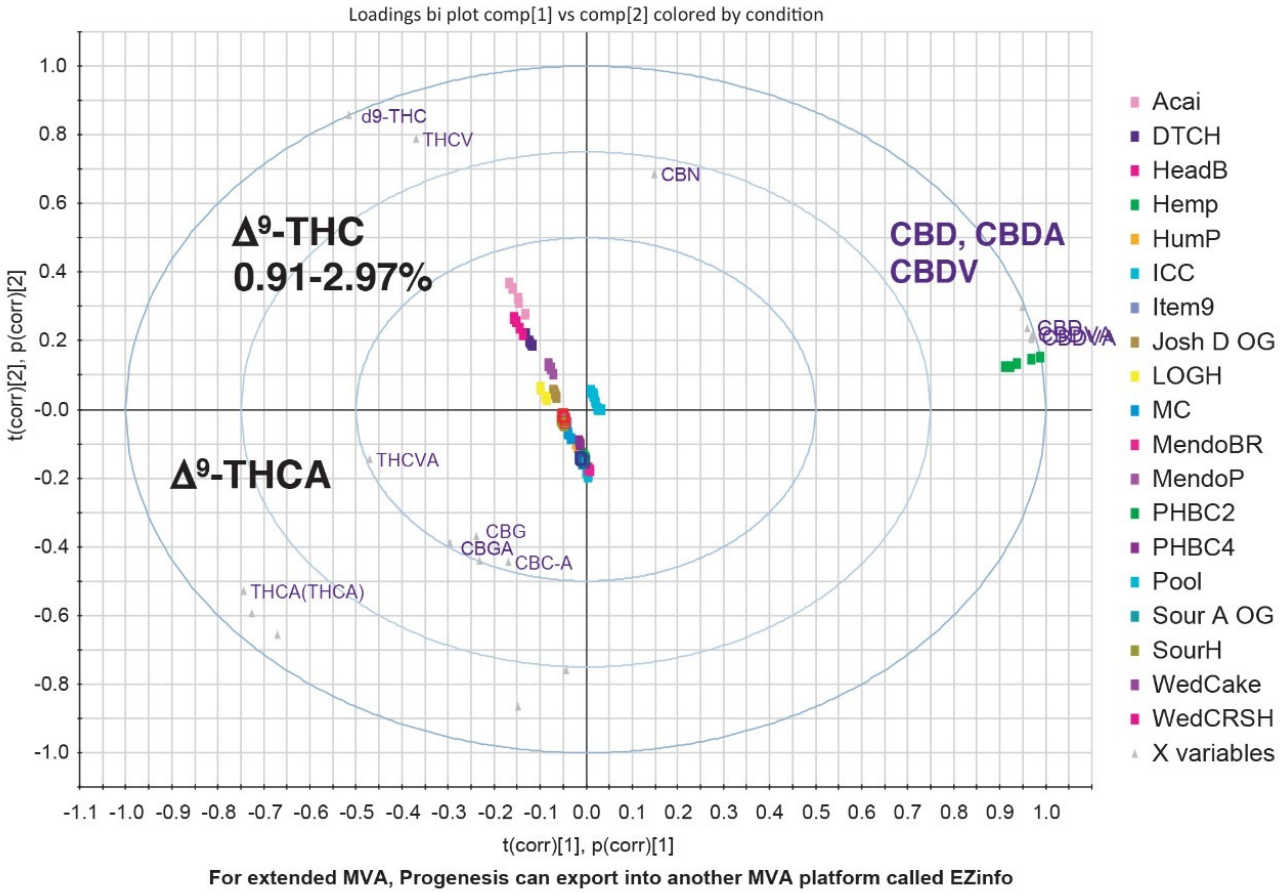


Figure 8. A loadings biplot plot showing the identified cannabinoid markers.

The loadings plot (A) can be used to interpret the patterns observed in the scores plot (B), which illustrates the relationships between the cultivars.[24] The greatest distinguishing chemical differences for the identified cannabinoids CBGA, THCA, and Δ^9-THC can be observed in the loadings plot. The cultivars that have greater expression of CBGA can be seen in the top right quadrant. Significant unknown markers of interest can be imported back into Progenesis QI. An unknown component 9.20_374.2463n, eluting at 9.20 minutes with a neutral mass of 374.2463 (highlighted by the red ellipse) was observed in the lower left quadrant in the direction of the Josh D OG cultivar (Figures 9A and B).

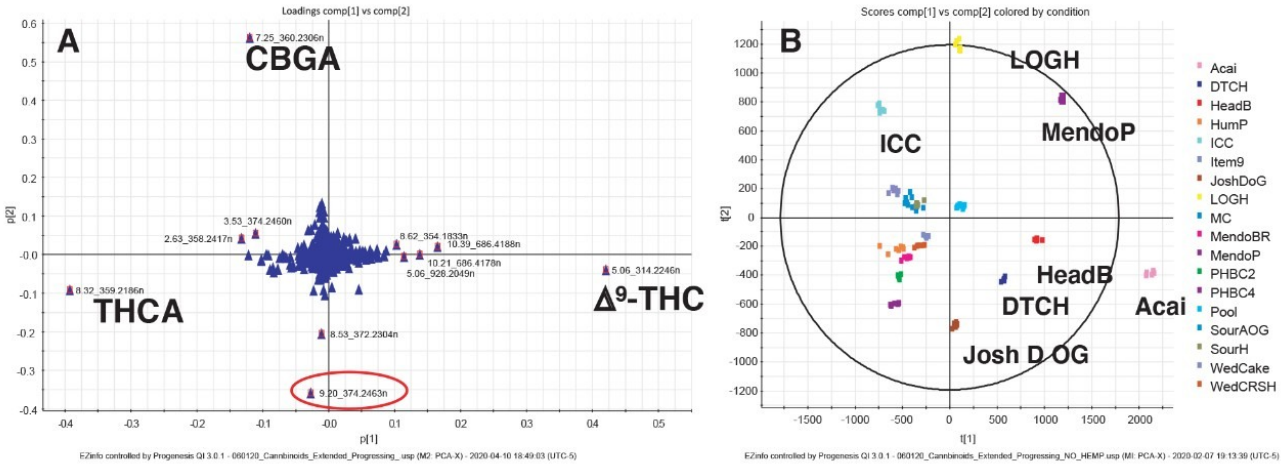
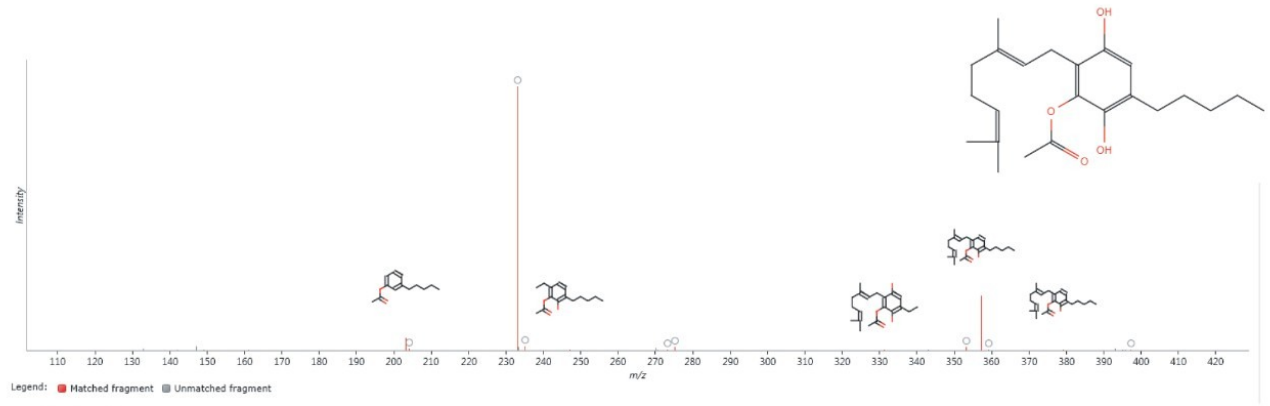


Figure 9. Loadings (A) and scores (B) plot showing the trends in the data when hemp is removed from the experiment.

The component was imported from the loadings plot in EZinfo into Progenesis QI for further evaluation. When a search was made for the marker against the in-house database, 4 compounds with the elemental composition $C_{23}H_{34}O_4$ were proposed and ranked based on the scores and the theoretical fragmentation performed for each individual proposal (Figure 10). Authentic standard compounds were not available to perform further confirmation of this identification. In separate negative ion ESI experiments, three major accurate mass fragments of this component (*m/z* 329.2486, $C_{22}H_{33}O_2$; *m/z* 245.1547, $C_{16}H_{21}O_2$; *m/z* 191.1077, $C_{12}H_{15}O_2$) were observed (data not shown) and could be matched with a previously reported unnamed cannabinoid of the same reported elemental composition.[14]



Possible identifications: 4

	Compound ID	D	Adducts	Formula	Retention time	Score	Fragmentation score	Mass error (ppm)	Retention time error (mins)	Isotope similarity
☆	4-acetoxy-2-geranyl-5-hydroxy-3-n-pentylphenol	4-	M+H, M+Na	$C_{23}H_{34}O_4$		G 54.4	G 76.6	0.51		95.97
★	5-acetyl-4-hydroxycannabigerol	5-	M+H, M+Na	$C_{23}H_{34}O_4$		H 54.4	H 76.6	0.51		95.97
☆	- trans-CBT-OEt-C5	- t	M+H, M+Na	$C_{23}H_{34}O_4$		G 48.9	G 49	0.51		95.97
☆	5-methoxycannabigerolic acid	5-	M+H, M+Na	$C_{23}H_{34}O_4$		G 48.2	G 45.7	0.51		95.97

Figure 10. Search results from the cannabinoid database for components with the elemental composition $C_{23}H_{34}O_4$.

The abundance profile for this component is shown in Figure 11. The max fold change was 409 with the highest mean detected in JoshD OG cultivar and the lowest mean in the HumP cultivar.

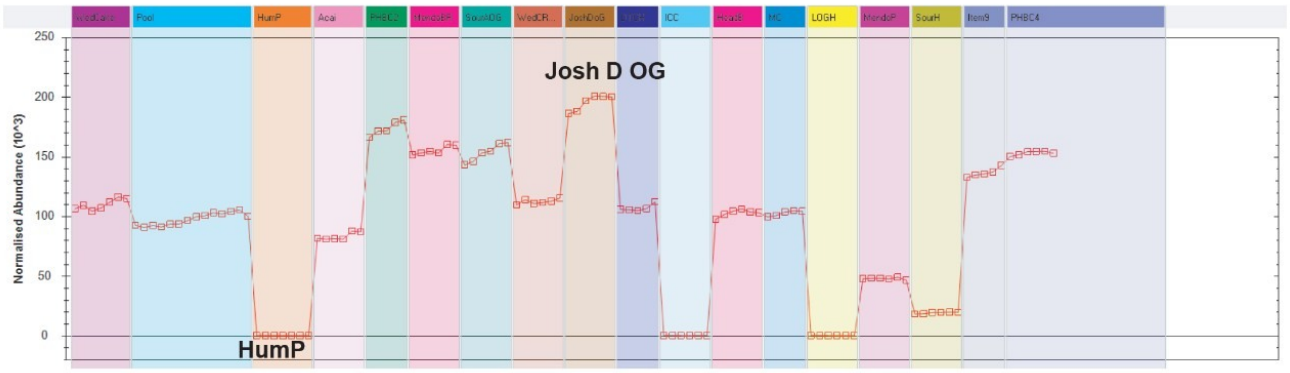


Figure 11. Abundance profile plot showing the difference in the relative behavior of unknown component 9.20_374.2463n across the test samples.

Additional significant components highlighted in the loadings data included CBNA at 8.62 min with a neutral mass of 354.1833n which was subsequently confirmed using an authentic standard. A component eluting at 8.53 minutes with a neutral mass of 372.2304n and a proposed elemental composition of $C_{23}H_{32}O_4$ was tentatively identified using the database entries as 2-acetoxycannbichromene.[8,28] The proposals were made based on the database entries available. Unambiguous identification would require verification using authentic standards or

further study including isolation followed by NMR.

Since cannabis cultivars vary in their chemical profiles, component target lists can be compiled within the Progenesis QI Software and used for subsequent targeted identification and differentiation.

APGC-Tof-MS Analysis of Terpenes

Terpenes have been reported to be exert a significant influence on the characteristics and effects of cannabis while also participating in many biological functions.[15,29] Chemotaxonomic discrimination of cannabis cultivars using terpene profiles have been attempted previously.[13,15-21]

The same workflow that was used to characterize the cannabinoid variation was also used for the terpenes. A GC separation method was developed to resolve the monoterpenes from the sesquiterpenes (Figure 12).[30] Information from the analysis of the authentic terpene standards was used to generate fragmentation and retention time databases that were subsequently used in conjunction with the structure database to increase the confidence in identification.

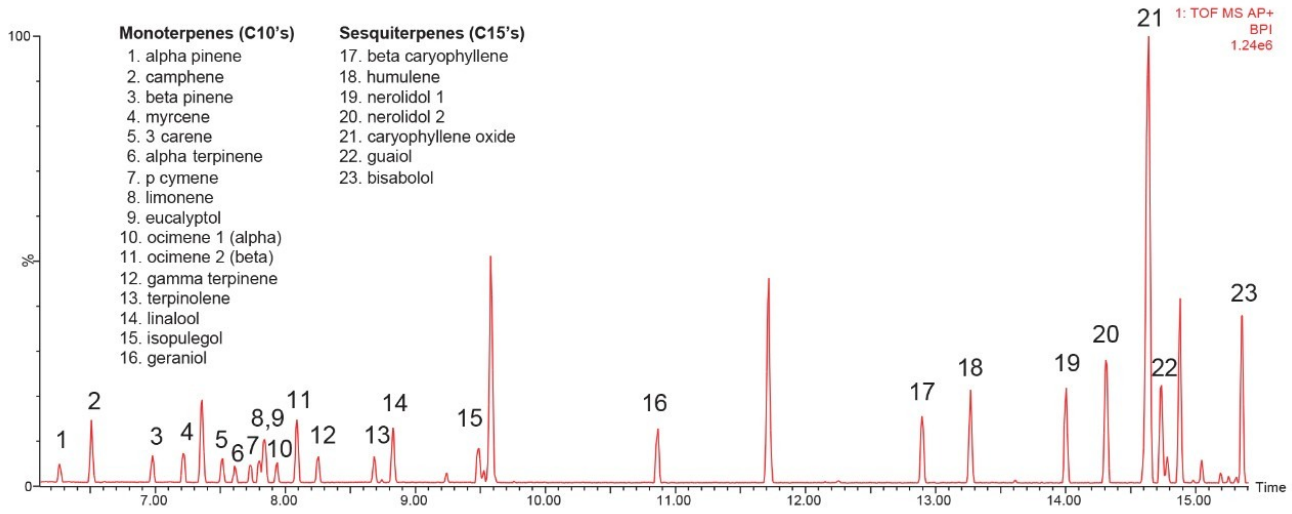


Figure 12. Base peak intensity chromatogram (BPI) of authentic standard mix of 23 terpenes analyzed in the study, 1 µL injection).

The cultivars LOGH, HumP, ICC, Acai, Hemp, Mendo P, and SourAOG cluster into chemically distinct groups in the PCA data (Figure 13). The loadings plot can be used to explain the differences in the distinct groups (data not shown).

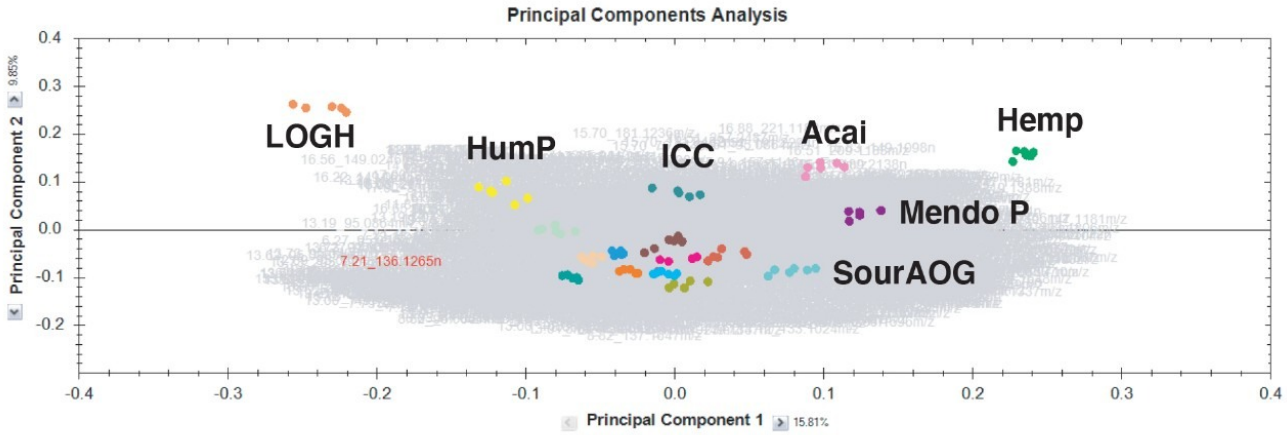


Figure 13. PCA plot for the APGC-MS analysis of cannabis cultivars.

In the Review Compounds window, the information about the target terpenes which were identified using the library are presented. The expression of β-myrcene was highest in the SourH cultivar and lowest in hemp (Figure 14).

Figure 14. Review Compounds provides a summary of data for the identified terpenes and other features.

Correlation analysis provides a means to separate samples into groups that share common properties facilitating ways to evaluate the relationships between the abundance profiles for the terpene/cannabinoids in the extracted cannabis samples. Correlations between the abundance profiles of β-Pinene, β-Myrcene, and α-Pinene were observed in the HumP, HeadB, LOGH, and SourH cultivars (Figure 15).

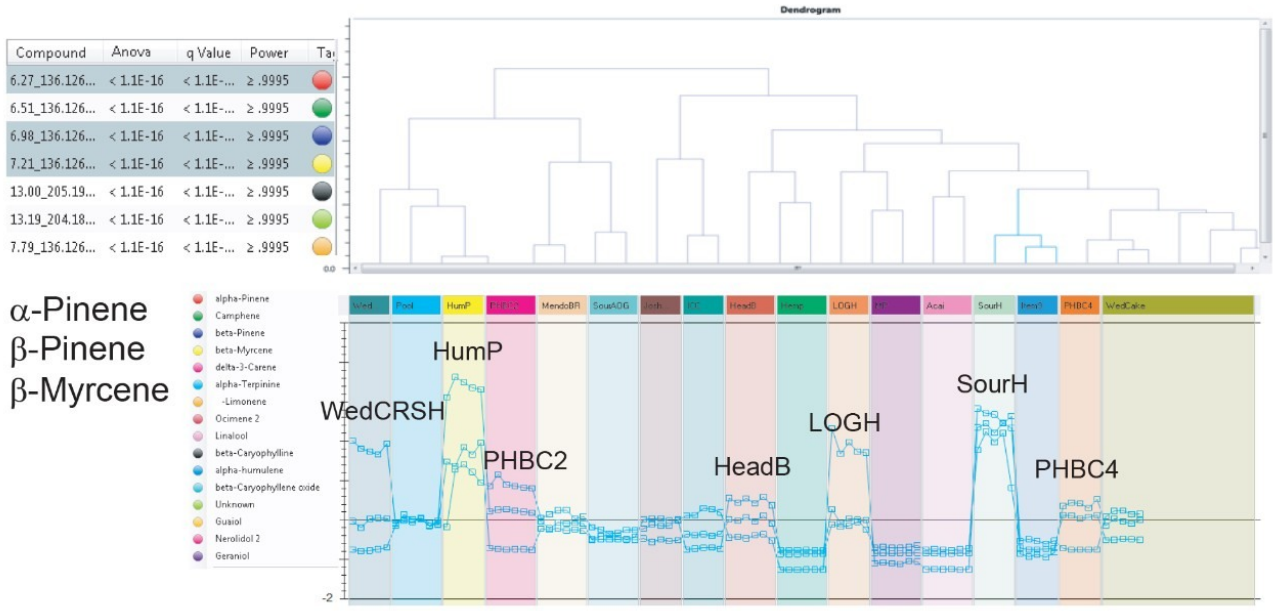


Figure 15. Correlation analysis showing similarities of the standardized abundance profiles for β-Pinene, β-Myrcene, and α-Pinene across the cannabis cultivars.

The loadings biplot shows selected terpene markers with stronger associations to the cultivars on the right side of the plot which is the opposite side of the plot to the Hemp group. The LOGH, SourH, and MendoBr cultivars are more strongly associated with the monoterpenes, β-Pinene, β-Ocimene, α-Terpinene, γ-Terpinene, terpinolene, and d-3-Carene (Figure 16). The HumP cultivar had greater expression for the terpenes β-myrcene, α-pinene, and caryophyllene oxide.

Retention time (min)	Accepted ID
6.98	beta-Pinene
7.51	delta-3-Carene
7.61	alpha-Terpinolene
8.09	beta-Ocimene
8.26	gamma Terpinolene
8.69	Terpinolene

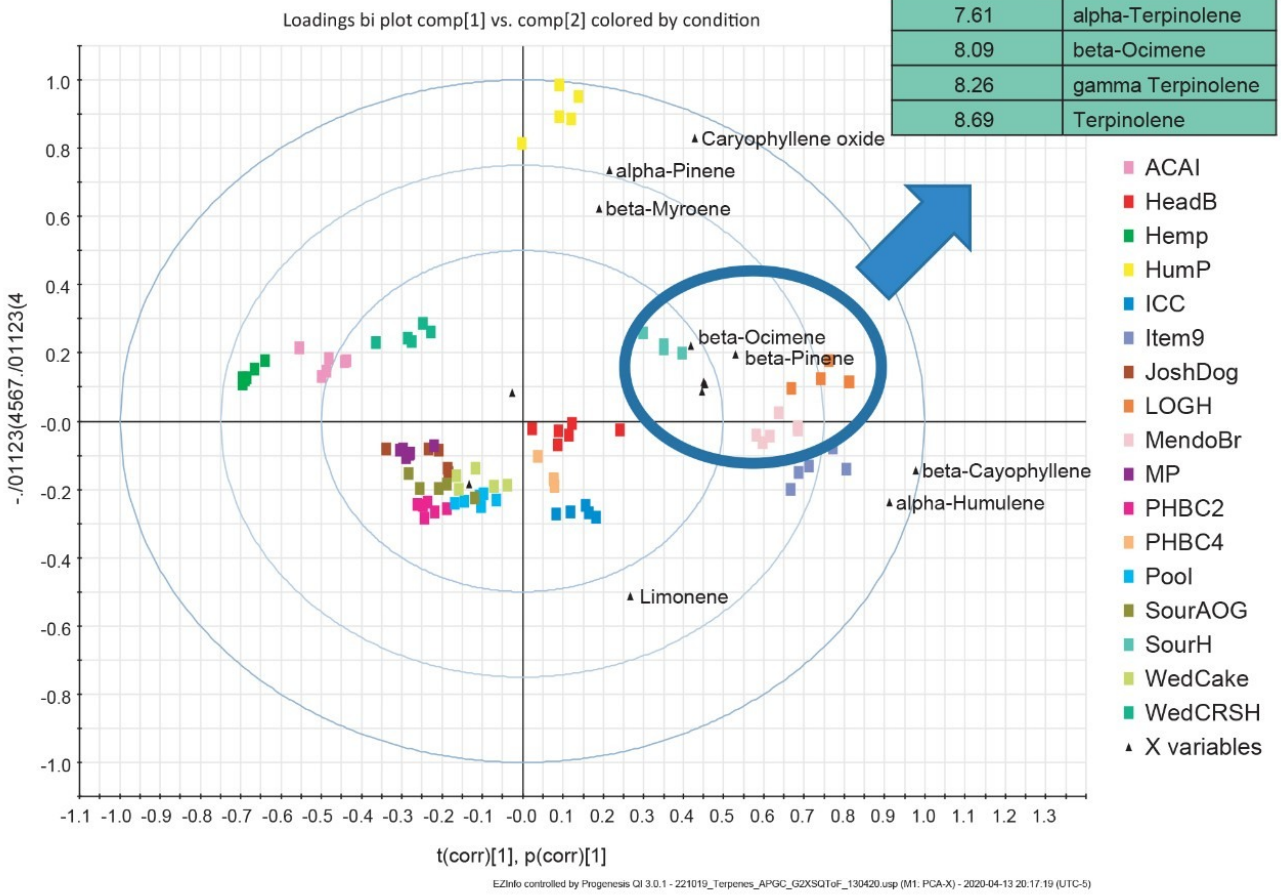


Figure 16. A loadings biplot showing select terpene markers.

The loadings data from the UPLC-Tof/MS and APGC-Tof/MS can be interpreted to identify correlations between the cannabinoid and terpene profiles. The data can be exported from Progenesis QI for further processing.

Conclusion

Differences between cultivars can be easily identified using UPLC-Tof/MS and APGC-Tof/MS coupled with Progenesis QI PCA data processing. The hemp cultivar showed the typical elevated levels of CBD and associated metabolites and low levels of Δ^9-THC. Cultivars with higher expression for Δ^9-THC were observed in the PCA. Exporting the data to EZ info provides access to extended MVA which can be used to interpret and explain the

chemical differences observed in the cultivars. Cannabinoid and terpene abundance profiles and hierarchical clustering dendrograms provide clear visualization of trends across the cultivars. Significant unknown components can be highlighted using MVA and sent to Progenesis QI for further interrogation.

A custom database greatly aided in the identification of the cannabinoids and terpenes detected from the analysis of the cultivars. The database consisted of compound structures, accurate mass as well as additional properties such as retention time and fragmentation which were created based on available authentic standards. Component identification was based on multiple attributes including retention time, accurate mass precursors, and fragmentation patterns as well as isotopic distributions, increasing confidence in the assignments made. Additional cannabinoids and terpene structural files can be added to the database to facilitate screening of larger numbers of analytes. Thirteen cannabinoids for which retention time and fragmentation spectra had been recorded were successfully identified using the library however the identities of many more were tentatively assigned based on the structural database screening and theoretical fragmentation, though authentic standards were not available for further confirmation. The generation of libraries and standardized chromatographic separations will allow improved characterization of the chemical diversity within the cannabis matrix.

These tools will allow scientists to analyze large sample sets under controlled analytical conditions, minimizing the effects of technical variation in the measurement and enabling them to evaluate multiple conditions, such as variations in growth environments or harvesting techniques, where relatively minor changes in the expression of cannabinoids and terpenes are expected to be observed.

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