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In-Depth Cannabis Multiclass Metabolite Profiling Using Sorptive Extraction and Multidimensional Gas Chromatography with Low- and High-Resolution Mass Spectrometry

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ABSTRACT: The present research reports on the development of a methodology to unravel the complex phytochemistry of cannabis. Specifically, cannabis inflorescences were considered and stir bar sorptive extraction (SBSE) was used for pre-concentration of the metabolites. Analytes were thermally-desorbed into a comprehensive two-dimensional gas chromatography (GC×GC) system coupled with low- and high-resolution mass spectrometry (MS). Particular attention was devoted to the optimization of the extraction conditions to extend the analytes' coverage, and to the optimization of the chromatographic separation to obtain a robust dataset for further untargeted analysis. Monoterpenes, sesquiterpenes, hydrocarbons, cannabinoids, other terpenoids, and fatty acids were considered to optimize the extraction conditions. The response of selected ions for each chemical class, delimited in specific 2D chromatographic regions, enabled an accurate and fast evaluation of the extraction variables (*i.e.*, time, temperature, solvent, salt addition), which were then selected to have a wide analyte selection and good reproducibility. Under optimized SBSE conditions, eight different cannabis inflorescences, and a quality control sample were analyzed and processed following an untargeted and unsupervised approach. Principal component analysis on all detected metabolites revealed chemical differences among the sample types which could be associated with the plant subspecies. With the same SBSE-GC×GC-MS methodology, a quantitative targeted analysis was performed on three common cannabinoids, namely Δ9-tetrahydrocannabinol, cannabidiol, and cannabinol. The method was validated, giving correlation factors over 0.993, and <12% reproducibility (relative standard deviation) at the lowest calibration point. The high-resolution MS acquisition allowed for high confidence identification and post-targeted analysis, confirming the presence of two pesticides, a plasticizer, and a cannabidiol degradation product in some of the samples.

Cannabis, intended as the botanical parts of the *Cannabis sativa L.* plant and its products, has been used for medical and recreational purposes for millennia.[1] Even though direction towards acceptance of cannabis has been slow due to regulatory and cultural issues, in the near future it is expected to play an important role in a considerable portion of the industry market strategies.[2] The increase of liberalization and decriminalization programs in the last decade has paved the way for the creation of new markets and the appearance of many new cannabis-related products can be observed, including foods, personal-care and medicinal formulations.[3]

Although more than 700 different cultivars have already been described,[4,5] the most common way currently used to classify cannabis cultivars is through plant morphology (phenotype) with two types typically recognized: *Cannabis sativa* spp. *sativa* and spp. *indica*. [6]

Far from the initial and simplistic consideration of only THC (Δ9-tetrahydrocannabinol) carrying the biological activity, many studies demonstrated the importance and the synergy of the surrounding diverse metabolites, progressing to the discovery and isolation of novel active compounds.[7] For example, very recently a new cannabinoid with a high potential of pharmacological effects was discovered and characterized.[8]

Many other endogenous metabolites are naturally present in the plant, and even though some single metabolites have demonstrated stronger activity than others, it is their balance which makes the final use more effective.[9,10] This consideration concerns not only the medical landscape for patients, but also the immense commercial scenario for regular customers, for example for edible products or cosmetics. Independently from the intended use, the initial plant chemical composition is important for the characteristics of the final products.

The phytochemistry of cannabis is complex, and more than 530 compounds have been identified,[11] unevenly distributed and belonging to different chemical classes and originating from primary and secondary metabolism.[9] Among them, approximately 110 were characterized as cannabinoids and 140 as terpenoids.[12] These two groups have raised special interest due to their organoleptic properties,[2] potential for chemically fingerprinting different cultivars,[13] and synergistic interactions with the cannabinoids.[10]

The study of cannabis composition dates quite far back,[14,15] and classical characterization approaches nowadays are not designed to fully map out such complex chemical diversity and heterogeneity among different cannabis types and products.[16–21] Many different subtypes of cannabis are known to exist, and the high number of (potential) active components significantly complicates conventional reductionist approaches.[4,7]

Recently, also the United States Pharmacopeia (USP) has reported on the classification of cannabis inflorescence, highlighting the importance and needs of better sampling procedures, analytical tests, and acceptance criteria to define the identity, content, and limits of the constituents.[22] Three main chemotypes of cannabis were identified (i.e. THC-dominant, cannabidiol (CBD)-dominant, and THC-CBD intermediate, which can be further sub-grouped based on the profile of other cannabinoids, terpenes or other constituents. A comprehensive overview of such a chemical diversity and a better classification of cannabis varieties would certainly

promote further implementation of cannabis-based products into clinical research and modern (personalized) medicine, but also provide more controlled and safe products for other use (food, cosmetics, *etc*.). Such a concept fits perfectly with the approach of metabolomics, which ideally aims to comprehensively detect, characterize, and quantify all metabolites in a biological system and then use multivariate data analysis to create a map of chemical diversity.[17,23]

The present research reports the multiclass and small metabolite profiling of cannabis. For this purpose, cannabis inflorescences were considered and stir bar sorptive extraction (SBSE) was used for pre-concentration of the metabolites.[24] Analytes were thermally-desorbed into a comprehensive two-dimensional gas chromatography (GC×GC) system coupled to both low- (LR) and high-resolution (HR) time-of-flight mass spectrometry (ToF MS).[25] Particular attention was devoted to the optimization of the extraction conditions to extend the analytes' coverage, and optimization of the chromatographic separation to obtain a reliable dataset for further untargeted analysis. Simultaneously, using the same methodology, a quantitative analysis was performed on the three regulated cannabinoids, namely THC, CBD, and cannabinol (CBN). The HR ToF capabilities were instead exploited for post-targeted analysis, confirming the presence of two pesticides, a plasticizer, and a cannabidiol degradation product in some of the samples.

EXPERIMENTAL SECTION

Samples and standards. Eight different types of cannabis flowers were purchased in a local CBD store (Liège, Belgium). Of these, two were reported as *indica*, four as *sativa*, and two as hybrid forms. Triplicates were prepared for each flower type. Samples were homogenized and stored at 20 °C in hermetic vials. For the optimization of the extraction conditions using design-of-experiment (DoE), a pooled sample (optimization sample) was used, constituted by mixing the eight different inflorescence types. The acidity of the solutions were measured, resulting with pH=4-6.

A hemp tea sample (dry leaves), was used for quality control (QC) during sample analyses. The QC sample underwent the same processing as for the cannabis dry inflorescences. For quantitative analysis, a sample of dry hop was used as surrogate matrix and was spiked before the extraction with the standard acid forms of the target cannabinoids to build calibration curves. Cannabidiolic acid (CBD-A), Δ9-tetrahydrocannabinolic acid A (THC-A) and CBN, were obtained from Restek Corporation (Bellefonte, PA, USA), and 5-point calibration curves (0.1, 0.2, 0.48, 1, 2 µg/mL for THC-A and CBN, and 1, 2, 4.8, 10, 20 µg/mL for CBD-A) were constructed using the surrogate matrix, and each point analyzed in triplicate using the final SBSE-GC×GC-MS conditions.

For the samples resulting with concentration over the calibration limit, additional extractions were obtained and analyzed using different split flow ratio.

A $C_{7\text{-}30}$ *n*-alkane mixture was used for retention index (RI) calculation and for modulation optimization, and was purchased from MilliporeSigma (Bellefonte, PA, USA). A standard mixture containing 22 terpenes, was supplied by Restek Corporation. Deuterated (D_5) chlorobenzene (Restek Corporation) was used as internal standard, was diluted in methanol, and present in the sample solutions at a final concentration of 0.1 µg/mL. The list of the 27 standard compounds (excluding the linear alkanes) and related information are reported in **Supplementary Table S1**. All solvents used were of HPLC grade.

Stir bar sorptive extraction and thermal desorption. Stir bars (Twister®) coated with 63 µL PDMS (polydimethylsiloxane, 10 mm length × 1.0 mm thickness) were obtained from Gerstel KK (Tokyo, Japan). The extraction parameters and levels listed in **Table 1** were optimized for the efficient multiclass metabolites' extraction of cannabis flowers (the final conditions are highlighted in bold). A fractional factorial DoE was used on the pooled samples to obtain 16 different conditions, which were run in triplicate, and the results visualized as main effect plots. The final conditions used for sample extraction consisted of 50 mg of dry inflorescence in 5 mL of a mixture water:methanol:acetone (5:4:1), for 60 min, at 50 °C. After each cycle, the stir bars were conditioned as advised by the manufacturer, and transferred to desorption tubes. Periodic random blank analyses confirmed the cleanliness of stir bars prior to extraction (data not reported).

GC×GC-LR ToF MS analysis. The principal system used for the study was a Pegasus 4D (LECO Corporation, St. Joseph, MI, USA) GC×GC LR ToF MS instrument with an Agilent 7890 GC equipped with a thermal desorption unit (TDU), cooled injection system (CIS), and a MultiPurposeSampler autosampler (Gerstel K.K.). Modulation occurred by means of a differential flow modulator in a symmetrical configuration. Briefly, the lab-made modulator was constructed by using two MXT Y-unions (Restek Corporation) and a 3-way solenoid valve (located outside the GC system), connected to an auxiliary pressure source. The two unions were bridged by using a deactivated capillary of 20 cm × 0.51 mm id, with this acting as an accumulation loop. More details on its performance and characteristics can be found in the literature.[26–29] The first dimension (^{1}D) column was a non-polar Rxi-5MS (5% diphenyl-95% dimethylpolysiloxane phase) of dimensions 30 m × 0.25 mm id × 0.25 µm d_f. The second dimension (^{2}D) column was a mid-polar Rxi-17Sil MS (equivalent to a 50% diphenyl-50% dimethylpolysiloxane phase) of dimensions 5.0 m × 0.25 mm id × 0.25 µm d_f. (Restek Corporation). The carrier gas was helium and the optimized column flow conditions were 0.4 and 7 mL/min, in the ^{1}D and ^{2}D, respectively.

The initial temperature of the TDU was set at 30 °C then heated to 300 °C (held 5 min) at 11.6 °C/s. The interface temperature was kept at 310 °C. The analytes were desorbed from the TDU in splitless mode and were focused at 20 °C on a glass liner packed with Tenax®. The injector was programmed from 20 °C to 300 °C (held 2 min) at 12 °C/s, and the injection was performed in the split mode (1:50). The primary and secondary oven temperature program was the same and started at 50 °C (held 2 min), then ramped to 330 °C (held 2 min) with a rate of 3 °C/min. The modulation period (P_M) was 6.6 s, consisting of an accumulation and reinjection time of 6 and 0.6 s, respectively. The unmodulated GC-MS profiles (**Supplementary Figure S2**) were acquired switching off the modulation timer and maintaining the same GC×GC-MS flow and temperature conditions.

A mass range of 40 to 400 *m/z* was monitored at an acquisition rate of 150 spectra/s, using electron ionization (70 eV). The ion source was maintained at 230 °C. Data

acquisition, alignment, and processing were performed using ChromaTOF® (LECO Corp., v. 4.72).

For peak detection, a signal-to-noise (S/N) cutoff was set at 50, and detected peaks were tentatively identified by a forward search using the NIST 2017 database (70% minimum similarity was required) and using retention index information (a ±20 RI tolerance window was considered). The reference linear retention indices on the non-polar column were extrapolated from AromaOffice® (Gerstel K.K., v.4), literature research, and the NIST 2017 database. For the peak alignment across chromatograms, maximum $^{1}t_R$ and $^{2}t_R$ (retention time in the first and second dimension) deviations were set at ±12 s and ±0.1 s, respectively, and the inter-chromatogram spectral match threshold was set at 70%. Moreover, the search for peaks not found by the initial peak finding during the alignment was set to 20 S/N, and detected peaks were checked to exclude artifacts from the stir bar or columns (*e.g.*, siloxanes). The analyses for the DoE extraction optimization, for the untargeted profiling on the samples, for the method validation, and the decarboxylation test were carried out on the flow modulated SBSE-GC×GC-LR ToF MS system.

The decarboxylation test for CBD-A and THC-A was obtained separately spiking 1 µL (100 µg/mL) of the acid and neutral forms on the surface of the SBSE (5 replicates). The SBSE were left absorbing the standards for 60 min and then placed into an empty TD glass tube for the analysis following the same SBSE-GC×GC-LR ToF MS method as described above.

GC×GC-HR ToF MS analysis. Single analyses for each sample type were also performed with a separate cryogenically modulated GC×GC system coupled with high-resolution ToF MS (mass resolution ≥ 25,000 fwhm). The GC×GC-HR ToF MS system (Leco Corporation) equipped with a TD100-xr thermal desorber unit (Markes International Ltd, Llantrisant, UK). The GC was equipped with a quad jet cryogenic modulator and a modulation period of 4 s (alternating 1.2 s hot and 0.8 s cold) was used. As for the GC×GC-LR ToF MS injections, the same column phase combination was used: the first-dimension column was a non-polar Rxi-5MS (5% diphenyl-95% dimethylpolysiloxane, Restek Corporation) of 30 m × 0.25 mm id × 0.25 µm d_f; the second-dimension column was mid-polar Rxi-17Sil MS (equivalent to a 50% diphenyl-50% dimethylpolysiloxane, Restek Corporation) of 2 m × 0.25 mm id × 0.25 µm d_f. The carrier gas was helium at 1 mL/min. The main GC oven was set to an initial temperature of 50 °C, held for 2 min, and ramped to final temperature of 330 °C at a rate of 5°C/min (held 2 min). The secondary oven followed the same temperature ramp, with a positive offset of 20 °C. The initial temperature for desorption was set at 50 °C (held 0.5 min) then heated to 300 °C (held 5 min). Analytes were desorbed and focused at 20 °C on the trap. The trap was programmed from 20 °C to 300 °C (held 2 min), and the injection was performed in split mode (1:50). The samples were analyzed in the high-resolution mode [mass resolution ≥ 25,000 (fwhm)], and a mass range of 40 to 500 *m/z* was collected at a rate of 150 spectra/s. The ion source and the transfer line were maintained at 250 °C. For data acquisition and data processing, ChromaTOF® for HRT (Leco Corporation, v. 4.2.3) was used.

Statistical analysis. In the DoE, the EICs (extracted ion currents) of the chemical classes and the IS (**Table 2**) were used to generate the main effect plot, using Minitab LLC (State College, PA, USA). Pareto charts were used to determine the magnitude and the importance of the effects of the variables, and ANOVA was used to consider the statistical significance (data not shown). For the untargeted profiling, unique *m/z* values for peak areas were used for the entire data processing. A frequency of observation criterion was applied to use the most consistent features, meaning positive identification in 75% of the replicates within each sample type (2/3 of the replicates for each sample group or 6/8 of the replicates for the QC). Statistical analyses and figures were obtained using R v.3.4.3 (R Foundation for Statistical Computing, Vienna, Austria). The only data pre-treatment on the raw data involved the necessary scaling step for data visualization: auto-scaling was used to carry out principle component analysis (PCA), heat map and hierarchical clustering analysis (HCA). The R package MetaboAnalyst was used to generate PCA and HCA plots. The statistical significance of metabolites was tested using non-parametric ANOVA multiclass test (Kruskal-Wallis), with a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Optimization of extraction conditions via experimental design. The SBSE approach has been historically exploited for trace contaminants analysis thanks to its high enrichment factor.[30] Only a few reports involving untargeted analysis using SBSE, coupled to conventional GC systems, have appeared.[31,32] The increased resolution power of multidimensional techniques has not been explored in combination with SBSE for untargeted analysis, but only for targeted contaminants.[33]

Here, SBSE was used with the intention to cover a wide volatility range, comprising higher molecular weight metabolites.[31] The PDMS-coated bars were immerged and stirred under controlled conditions in water-based solutions (5 mL) of the cannabis flowers. For the extraction conditions, a DoE was built to optimize the extraction, evaluating different levels of the variables (extr. time, extr. temp, solvent, and salt addition) at the same time, and finally determine the most favorable ones. This process was carried out using the optimization sample (pooled inflorescence samples). Solvent types, extraction time and temperature, and salt addition were selected as important variables in tuning the extraction.[24] These variables and their respective levels are reported in **Table 1**. To reduce the number of runs, a fractional factorial design was used resulting in a total of 16 different conditions (each injected in triplicate). Aiming for an untargeted and broad metabolic profiling, the final extraction conditions were chosen to cover a wide range of metabolites, with appropriate sensitivity.

Based on the structured GC×GC separation (**Figure 1**), clusters of analytes could be readily and visually observed in the 2D chromatogram, with each belonging to a specific chemical class. Six different elution areas were defined by $^{1}t_R$ and $^{2}t_R$ values. In addition to the elution behavior, the mass spectral information (LR ToF MS) and injections of standards (monoterpenes, sesquiterpenes, cannabinoids, linear hydrocarbons – **Supplementary Table S1**) were used to confirm the classification elution regions.

Table 1. Parameters and values tested for the extraction optimization using the DoE. The final conditions are in bold (W = water, M = methanol, A = acetone).

Parameters	Levels			
Extraction time	10 min	20 min	**60 min**	90 min
Extraction temperature	25 °C	35 °C	**50 °C**	-
Extraction solvents (*v/v*)	W/M (3:1)	**W/M/A (5:4:1)**	W/A (9:1)	-
Salt addition (NaCl, 10% *w/w*)	**No**	Yes	-	-

To evaluate the different extraction conditions, and thus the extraction efficiency, the response of the entire chemical class was considered. Specifically, a MS criterion was applied using the area generated from characteristic *m/z* ion(s) as response of the chemical class (see **Table 2**). For example, the EIC generated from the sum of the 231+295+299 *m/z* ions, and eluting in the classification region of the cannabinoids, was exploited for this class during the extraction optimization. The IS, added to the sample before the SBSE extraction, was evaluated as a single compound and the characteristic 117 *m/z* ion was monitored.

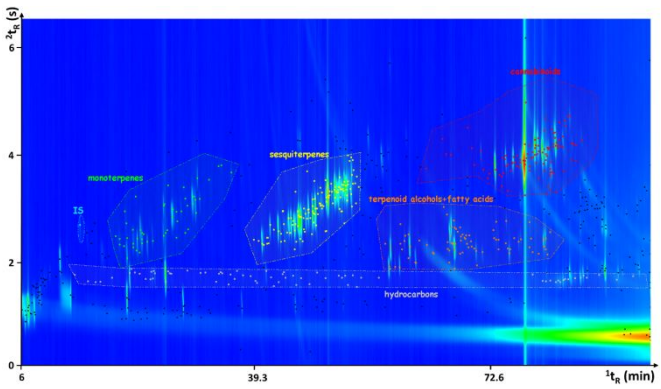

Figure 1. Representative 2D chromatogram of the SBSE-GC×GC-LR ToF MS analysis using unit duty-cycle flow modulation on cannabis inflorescence (sample 7). The classification regions for the chemical classes are highlighted (IS = internal standard).

For each class the combination of the elution classification region and the MS filter facilitated the selection of the extraction conditions, allowing I) an accurate signal evaluation over an extended number of analytes (~ 800) rather than a selected few, and more importantly II) it minimized misclassification errors. Therefore, in the case of chromatographically misclassified compounds, the MS response of their characteristic ions contributes minimally.
The response (EIC) generated from the 16 DoE conditions was plotted (**Figure 2**) in function of the variables (temp, time, solvents, salt addition) for each class (monoterpenes, sesquiterpenes, IS, hydrocarbons, cannabinoids, terpenoid alcohols and fatty acids).

Table 2. Chemical classes (and IS) and *m/z* ions used for DoE evaluation. The corresponding GC×GC elution regions are shown in Figure 1.

Chemical class	EIC (*m/z*)
Monoterpenes	93+91
Sesquiterpenes	93+91
IS (d_5-chlorobenzene)	117
Hydrocarbons	55+57
Cannabinoids	231+295+299
Terpenoid alcohols and fatty acids	58+68+71+74+79+88

Figure 2 summarizes the DoE results, and shows, for each class, the response trend under the different extraction variables and levels, which supported the selection of the final extraction conditions (highlighted in bold in **Table 1**).
The chemical classes under observation showed a general higher yield (qualitatively and quantitatively) with the increase of extraction time and temperature (90 min and 50° C). An exception to this was the IS, which exhibited an opposite trend. Also, the response of hydrocarbons appeared not to be affected by the change of the extraction temperature. Salt addition had a weak impact on the extraction yield. Given that the salt addition (NaCl, 10% *w/w*) represents an additional step in the sample preparation process and it did not significantly affect the extraction, it was excluded from the final conditions. More importantly, the extraction yield with the different solvent mixtures (W/A, W/M/A, W/M) appeared to be specific to the chemical class. The triphasic W/M/A solution accounted for a good compromise in terms of extraction yield and chemical class coverage.
With regards to extraction time, it was observed that for some chemical classes (*e.g.*, mono and sesquiterpenes), no prominent plateau was clearly visible after the 90 min extraction tested, meaning that the solute-PDMS partition equilibrium was not totally reached. However, given that the 60 min extraction time produced a satisfactory response and reproducibility, a non-equilibrium sampling time was chosen to stay within the GC×GC analysis time (93 min), allowing for high throughput analysis.
Based on these observations on the chemical classes of interest, the final SBSE conditions were set using the W/M/A solvent mixture for 60 min, at 50 °C, and without salt addition (**Table 1**). As visible in **Figure 2**, the final extraction conditions were not optimal for the IS, but its response was considered acceptable and it showed a satisfactory relative standard deviation (RSD) below 12% (**Supplementary Table S2**).

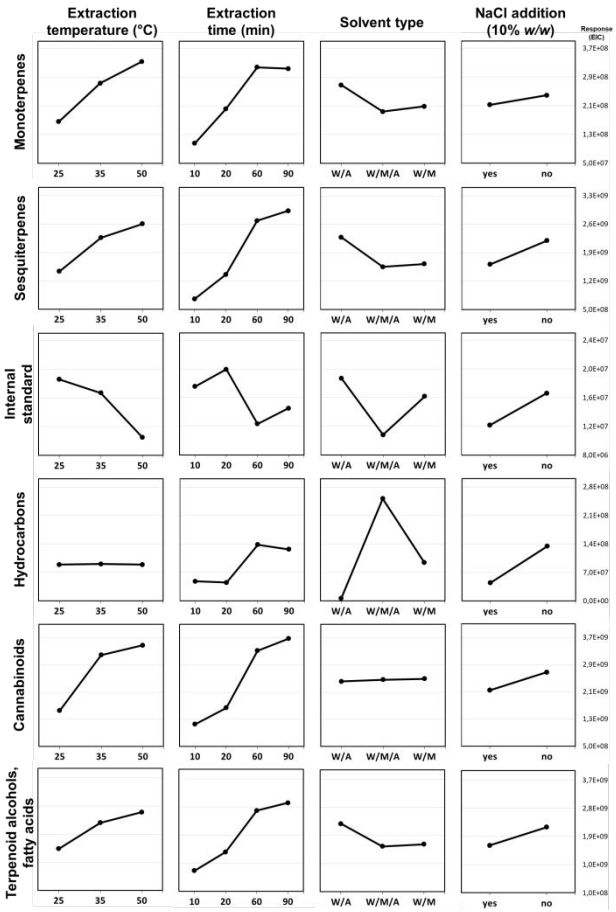

Figure 2. Main effect plot reporting the response under different extraction conditions (temp, time, solvent, salt addition) of monoterpenes, sesquiterpenes, IS, hydrocarbons, cannabinoids, terpenoid alcohols and fatty acids. W = water, M = methanol, A = acetone.

Profiling of Cannabis Metabolites. Comprehensive 2D GC is a powerful technique used for the resolution of complex samples, with only a few reports describing its application to the static headspace of cannabis inflorescences and resins or extracted *via* supercritical fluids and Soxhlet.[34,35] Typical advantages of the multidimensional techniques are the superior selectivity and signal enhancement compared to a conventional separation.[36,37] In this regard, **Supplementary Figure S1** shows the increased resolution and selectivity provided by the 2D separation on the cannabinoids region, and **Supplementary Figure S2** shows the signal enhancement provided by the modulation effect on the sesquiterpene region. In the present work, eight cannabis dry inflorescences (n=3) were extracted using the final SBSE conditions optimized via DoE (**Table 1** and **Figure 2**), and thermally desorbed in a flow-modulated GC×GC-MS instrument.[26,38] A hemp tea sample (n=8), similar to the inflorescences' chemical composition, was included in the analytical workflow. It was used as QC sample to monitor possible extraction variation and/or instrumental drift.

More than a thousand peaks were initially detected from the alignment of the 32 chromatograms (including the QC) using MS and chromatographic retention information (^1t_R and ^2t_R). The list of features was reduced to 754, retaining only those consistently detected (present minimum in 2/3 of the replicates) and filtering out chemical artifacts (from the stir bar or columns). This refined list of compounds was used further for the untargeted data analysis. A brief schematic of the entire data processing workflow is shown in **Supplementary Figure S3**.

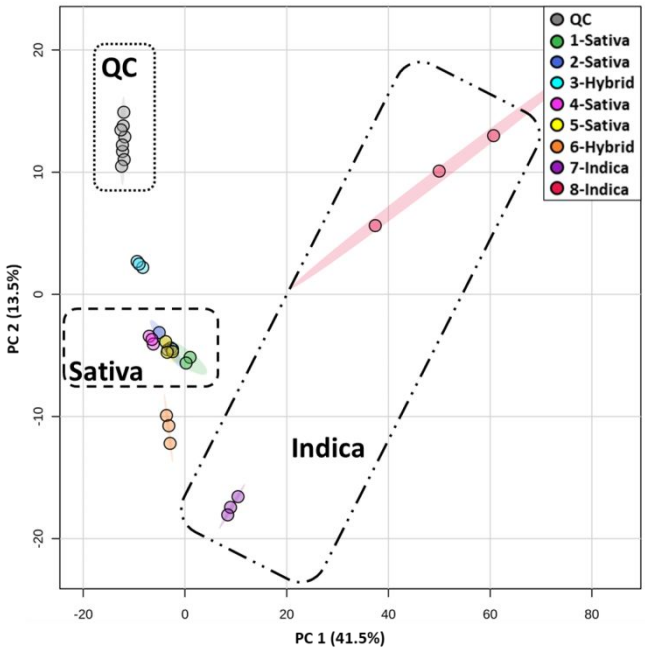

Figure 3. Untargeted PCA of the 754 features from the SBSE-GC×GC-MS untargeted analysis on cannabis inflorescences. Ellipses represent the 95% confidence interval for each group.

The untargeted and unsupervised data analysis approach, in combination with a minimal sample preparation (limiting the possibility of sample alteration) provided a comprehensive chemical profile of all the cannabis flowers under investigation.[39] Such a strategy, sustained by well-controlled analytical conditions, allowed for a minimal data manipulation, with only the auto-scaling being used as data pre-treatment for visualization purposes (PCA, HCA).

Figure 3 shows the untargeted PCA of the SBSE-GC×GC-MS of the samples, involving the 754 features. Tight clusters are formed based on the chemovar of the cannabis inflorescences. If the cannabis subspecies are considered, then the *sativa* samples (1, 2, 4, 5) result grouped together, separately from *indica* (samples 7, 8), and the hybrid ones (samples 3, 6). The fact that a clustering within replicates and within sample-types was obtained using such a nude data analysis workflow means that not only the analytical procedure is reproducible and robust, but that it contains the chemical information necessary to characterize and discriminate between the different samples. Indeed, the narrow cluster of the QC samples and the IS reproducibility (14% RSD considering all the injections) are an indication of the well-controlled overall methodology. The IS reproducibility was controlled *via* univariate control chart over time, and is reported in **Figure 4**. Considering the other chemical classes, the RSD of the response in the QC did not exceed 18% (**Supplementary Table S2**).

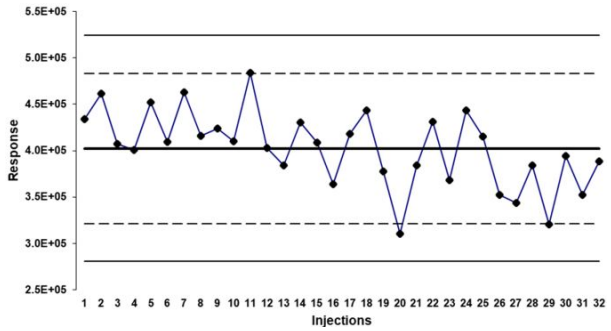

Figure 4. Control response chart of the internal standard in the cannabis and QC samples over time. The middle line represents the average value, the dotted lines represent the 2 σ and 3 σ lower and upper limits. A 14% RSD during the sample analyses was observed.

For a better visualization of the sample chemical map, 70 features were selected among the most statistically significant ones (Kruskal-Wallis, $p > 0.05$), and more expressed in at least one of the sample types. A heat map depicting the relative abundance of these cannabis metabolites is shown in **Figure 5**, with each column representing a single sample replicate, and each row representing a metabolite.

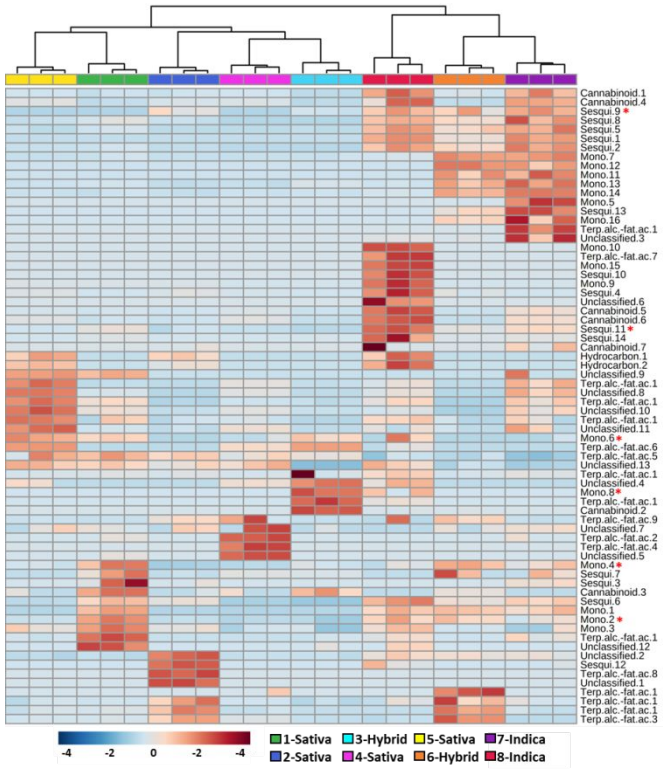

Figure 5. Heat map and hierarchical clustering analysis of 70 selected cannabis metabolites that were significantly different between the eight samples. The metabolites with * were confirmed *via* standard injection. The label "unclassified" represent the peaks eluting outside the classification regions in the GC×GC chromatogram.

The characteristic and significantly different features, classified on the basis of the GC×GC elution zones and MS information, consisted of 16 monoterpenes, 14 sesquiterpenes, 7 cannabinoids, 18 terpenoid alcohols and fatty acids, 2 hydrocarbons, and 13 unclassified compounds (p values ranging from 1.46×10^{-4} to 2.63×10^{-2}). These compounds represent well the elution classification groups, excluded the hydrocarbons which bear less informative and characteristic values compared to the other classes.

Using the selected metabolites, the discrimination of the cannabis subspecies is more evident in the dendrogram in **Figure 5,** with the two main branches which divide the *sativa* from the *indica*. In addition, a better clustering within the *sativa* spp. samples is possible (**Supplementary Figure S4**). Among the 70 selected metabolites, the following 6 terpenes (4 mono and 2 sesquiterpenes) were confirmed through injection of standards: α-pinene, β-myrcene, γ-terpinene, eucalyptol, nerolidol, and caryophyllene oxide. For more qualitative information of the 70 selected analytes, please refer to **Supplementary Table S3**.

In addition, and based on the recent recommendation from the USP, the samples were subclassified on the terpene relative dominance.[22] They fell under four categories, and specifically the β-caryophyllene dominance, the β-myrcene/β-caryophyllene, the limonene/β-caryophyllene, and the β-myrcene/limonene/β-caryophyllene co-dominance (**Supplementary Table S4**).

Analyte confirmation *via* HR MS. In parallel, single analyses for each sample was performed with a separate cryogenically-modulated GC×GC system coupled with high-resolution ToF MS (mass resolution ≥ 25,000 fwhm). High-resolution mass spectra can be highly beneficial in untargeted analysis, helping in the identification of unknowns and/or increasing the confidence of identification.[40]

In this study, the mass accuracy and selectivity of the HR MS system allowed the high confidence identification of some exogenous compounds, which were previously detected using the separate GC×GC-LR ToF MS system.

The pesticides chlorothalonil and cyprodinil, detected only in one sample (sample 7), were confirmed with a mass accuracy of 0.55 mDa and 0.02 mDa on the molecular (*m/z* 263.8810) and $[M\text{-}H]^+$ ions (*m/z* 224.1183), respectively. Interestingly, chlorothalonil is reported among the crop protection agents with a suggested limit of 1 µg/mL in tobacco and cannabis crops.[41,42] Cyprodinil is instead listed in Canada among the mandatory tested pesticides in cannabis, but the maximum residual limit has not been released yet.[43] For these, retention index values were calculated and matched with those present in the database for a non-polar column: RI values of 2072 and 1843 were calculated for cyprodinil (reference RI: 2068) and chlorothalonil (reference RI: 1822). The deviation from the reference values can be explained by the additional retention of the ^{2}D in the GC×GC system, obviously not considered in the databases.

The plasticizer additive bisphenol G was confirmed with a 0.28 mDa accuracy ($[M^{+\bullet}]$ *m/z* 312.2092), and it was detected in all the samples analyzed. Such a consistent contamination presumably derived from the plastic containers where the samples were sold and stored prior to the study.

Noteworthy, also the USP has recently highlighted that testing methodologies should also be available to confirm the absence of synthetic cannabinoids, which are an emerging issue and are particularly harmful.[22,44–46] The GC×GC-HR ToF MS system allowed for post-targeted analysis, and the samples were screened for the major synthetic cannabinoids and degradation products.[47] Among these, the cannabinoid

quinone HU-331, resulting from the oxidation of CBD,[48] was detected in one sample (sample 8), with a mass accuracy on the molecular ion (*m/z* 328.2038) of 0.59 mDa.
The experimental HR mass spectra for the aforementioned detected compounds are reported in **Supplementary Figure S5A-D**.
It is worthy to add that, even if the different modulation performances and characteristics produced different ^{1}D and ^{2}D retention times in flow- and cryogenically- modulated GC×GC chromatograms, the overall elution patterns were maintained in the two parallel systems thanks to the same column set used. In this situation, the retention time matching between the flow- and cryogenically-modulated systems could be possible *via* a fine experimental conditions optimization or software elaborations,[49,50] but it was beyond the scope of the current research.

Inlet decarboxylation of CBD-A and THC-A. The thermal lability of the acid forms of the cannabinoids is a known issue when using GC separation.[51] As results of the high inlet temperatures indeed, the native cannabinoids in the acid forms are subjected to decarboxylation into their neutral forms. For this reason, the GC determination of cannabinoids is unable to quantify acid and neural form separately and the total concentration (acid + neutral) can be reported. If the individual forms are sought to be determined, a LC-based method or a derivatization step prior GC analysis should be pursued.[20] The inlet conversion is usually not quantitative and it was tested here spiking the same amount of the neutral (CBD, THC) and the acid standard (CBD-A, THC-A), separately on different stir bars (n=5). The conversion of the acid to the neutral forms yielded 61% and 56%, though not affecting negatively the necessary method sensitivity for this study. **Supplementary Table S4** resumes the results of the inlet conversion yield.

Method validation for CBD, THC, and CBN. To demonstrate the capability of the SBSE-GC×GC-LR ToF MS approach for quantitative analysis, the overall method was validated by assessing the limit of detection (LOD), limit of quantitation (LOQ), linearity, precision, and accuracy for selected cannabinoids.
Due to the lack of a standard reference material, the matrix was simulated by using dry hop, as it belongs to the *cannabinaceae* family, and does not contain any significant levels of natural cannabinoids.[52] Calibrations curves were built on two concentration levels, in line with the natural distribution of the analytes in the samples, and specifically in the range of 0.1-2 µg/mL for THC-A and CBN, and 1-20 µg/mL for CBD-A. The highest point of the calibration curve corresponded to 0.2% *w/w* in the samples. Figures of merit of the method are summarized in **Table 3.**
The three target analytes showed a satisfactory analytical performance with good linearity over the calibration range (r^2 > 0.98), a reproducibility ranging from 9 to 19% RSD at the lowest point, and from 13 to 17% RDS at the highest point of the calibration curve. The accuracy, calculated in the low and high range of the calibration curve resulted satisfactory, with relative errors of THC below 10% for the concentration level close to the regulated limit in Europe (0.2% *w/w*).[53] The bias for THC and CBN resulted satisfactory at both low and high concentrations. The higher bias (35.6%) obtained for CBD at low concentrations (specifically at 0.14% *w/w*) was considered acceptable because of the well-known higher CBD concentrations in cannabis inflorescences, normally in the range 1-15% *w/w*.[7,54,55] The analyzed samples were all characterized by a total CBD concentration ≥ 1.5% *w/w*. Thus, the relevance of the high-end of the calibration curve here was more important for real-world sample quantification. However, for atypical low-CBD chemotypes or samples (*e.g.*, cannabis leaves, seeds, stems), an adapted calibration curve should be implemented/considered.

Table 3. Figures of merit for the SBSE-GC×GC-LR ToF MS method for the target compounds.

Cannabinoid	*m/z*	Reproducibility (RSD%)		Correlation coefficient (r^2)	LoD[c]	LoQ[c]	Accuracy (% bias)	
		low[a]	high[b]				low[d]	high[e]
CBD	231	8.8	12.7	0.9861	0.15	0.51	35.6	-11.7
THC	299	19.3	14.9	0.9848	0.03	0.11	20	-9.4
CBN	295	8.8	16.5	0.9839	0.02	0.05	-1.6	-1.8

[a] extrapolated at the lowest point of the calibration curve (0.1 µg/mL for THC-A and CBD and 1 µg/mL for CBD-A)
[b] extrapolated at the highest point of the calibration curve (2 µg/mL for THC-A and CBD and 20 µg/mL for CBD-A)
[c] expressed in µg/mL
[d] determined in hop spiked at 0.14 µg/mL (for THC-A and CBN) and 1.4 µg/mL (for CBD-A) level
[e] determined in hop spiked at 1.4 µg/mL (for THC-A and CBN) and 14 µg/mL (for CBD-A) level

The samples, as reported in the labels, did not exceed 0.2% *w/w* of total THC. The concentration of CBN, THC, and CBN determined in the samples are reposted in **Supplementary Table S5**. In addition, and referring to the very recent USP cannabinoid-chemotype classification, the samples fell in the CBD-dominant chemotype, with a ratio of total THC and total CBD not more than 0.2 and containing not less than 1% *w/w* total CBD and not more than 1% *w/w* total THC.[22]

Concluding Remarks

A comprehensive analytical method and workflow for the analysis of metabolites in cannabis inflorescences has been herein detailed. The method includes 1) sample extraction *via* SBSE, optimized to maintain a wide selectivity towards multiple classes of metabolites (terpenes, cannabinoids, hydrocarbons, terpenoid alcohols and fatty acids); 2) a high-resolution and affordable separation technique (GC×GC with flow modulation) suitable for the separation of hundreds of metabolites; 3) LR ToF MS detection, accompanied in this specific case by an HR ToF MS analyzer, to obtain qualitative and quantitative information of the detected metabolites. The information-dense dataset was handled with a neat multivariate untargeted analysis workflow to show the differences among the samples in accordance with the subspecies, allowing their classification based on the chemotype, following the recent USP recommendations.
The high sensitivity, wide selectivity and resolution of the overall methodology make it a powerful fingerprinting tool for detailed cannabis-related samples. The extraction method covered the terpene and cannabinoid classes, but also highlighted the importance of other phytocompounds (other terpenoid and fatty acids) in the classification of such cannabis samples. It must be affirmed that, if a targeted compound/class is sought, the extraction conditions can be tailored further to enhance a specific chemical class response.
Even if in-depth exogenous compound screening was outside the scope of the current research, the confirmation *via* GC×GC-HR ToF MS of the two pesticides, one plasticizer and one degradation product (the first three contaminants

were detected also through GC×GC-LR ToF MS), reinforces the concerns on the cannabis market regarding quality and safety. The method demonstrates the importance of novel comprehensive and high-resolution testing and characterization methods for cannabis-related products. This study represents the initial application of the method to cannabis inflorescences, and it will be extended to other related sample-types.

From a technical viewpoint, the use of SBSE, a green sample preparation technique, and flow-modulated GC×GC-MS, which does not require cryogens, are for the first time herein used in combination, and represented an effective global green approach for the untargeted profiling and targeted analysis of complex samples.

ASSOCIATED CONTENT

Supporting Information

Supplementary Table S1: Chemical class, compound name, CAS number (CAS#) and experimental retention index (RI_{exp}) of the 27 chemical standards used for chemical class elution classification in the GC×GC system and method validation.

Supplementary Table S2: Relative standard deviation (RSD%) of the chemical classes of interest in the quality control sample under the final SBSE conditions.

Supplementary Table S3: Qualitative information (MS library similarity (MS lib. match) and experimental retention index (RI_{exp})) of the compounds reported in Figure 5. For the retention index search, the tolerance was set to ±20 units. When a reference retention index was not available and high MS similarity (>800) was obtained, the MS library hit is reported.

Supplementary Table S4: Repeatability and inlet conversion yields (5 replicates) of the acid CBD-A and THC-A to the neutral forms CBD and THC.

Supplementary Table S5: Quantitative information and chemotyping of the analyzed samples. Quantitative values were obtained from 3 replicates and are expressed in *w/w*%.

Supplementary Figure S1: The 2D chromatogram expansion of the cannabinoids elution zone (sample 7) highlighting the selectivity of GC×GC. I) Separation between chromatographic artifacts (i.e. column bleed) and analytes of interest. II) Resolution of analyte pairs (arrows) which would co-elute in conventional 1D GC.

Supplementary Figure S2: Comparison of flow-modulated GC×GC (lower) and unmodulated GC (upper) traces showing the difference in response magnitude (sesquiterpenes elution zone of sample 8).

Supplementary Figure S3: Workflow of data treatment for the cannabis inflorescences analysis.

Supplementary Figure S4: PC scores plot of the selected 70 features from the cannabis inflorescences. Features were selected amongst the most statistically significant ones (Kruskal-Wallis, $p > 0.05$), and more expressed at least in one of the sample type. Ellipses represent the 95% confidence interval for each group.

Supplementary Figure S5A-D: High-resolution mass spectra (≥ 25,000 fwhm) of chlorothalonil (A), cyprodinil (B), bisphenol G (C), and HU-331 (D).

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Notes

The authors declare no competing financial interest.

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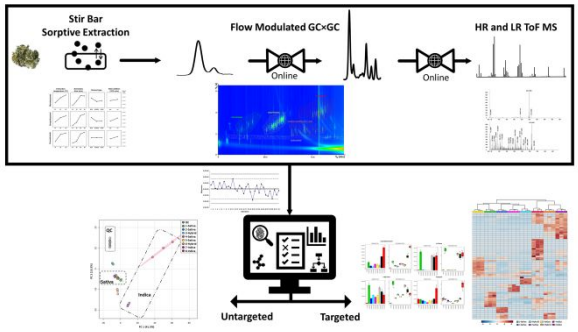
TOC



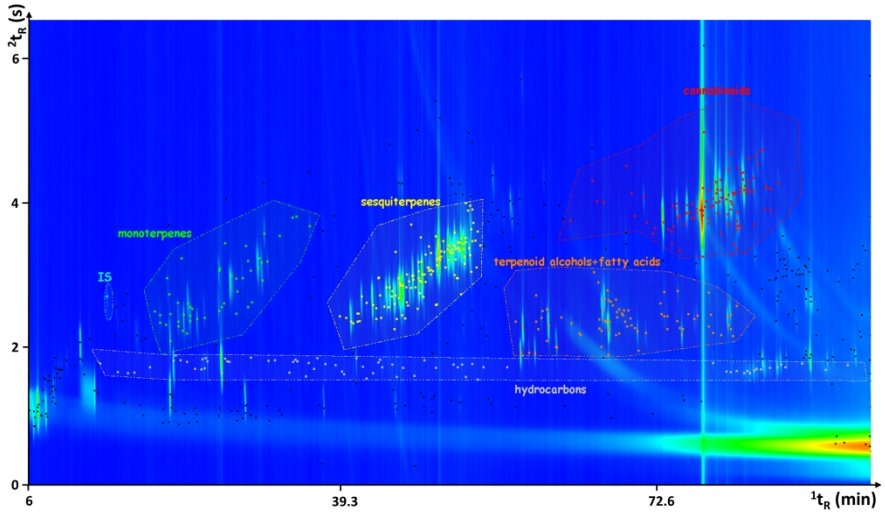


Figure 1. Representative 2D chromatogram of the SBSE-GC×GC-LR ToF MS analysis using unit duty-cycle flow modulation on cannabis inflorescence (sample 7). The classification regions for the chemical classes are highlighted (IS = internal standard).

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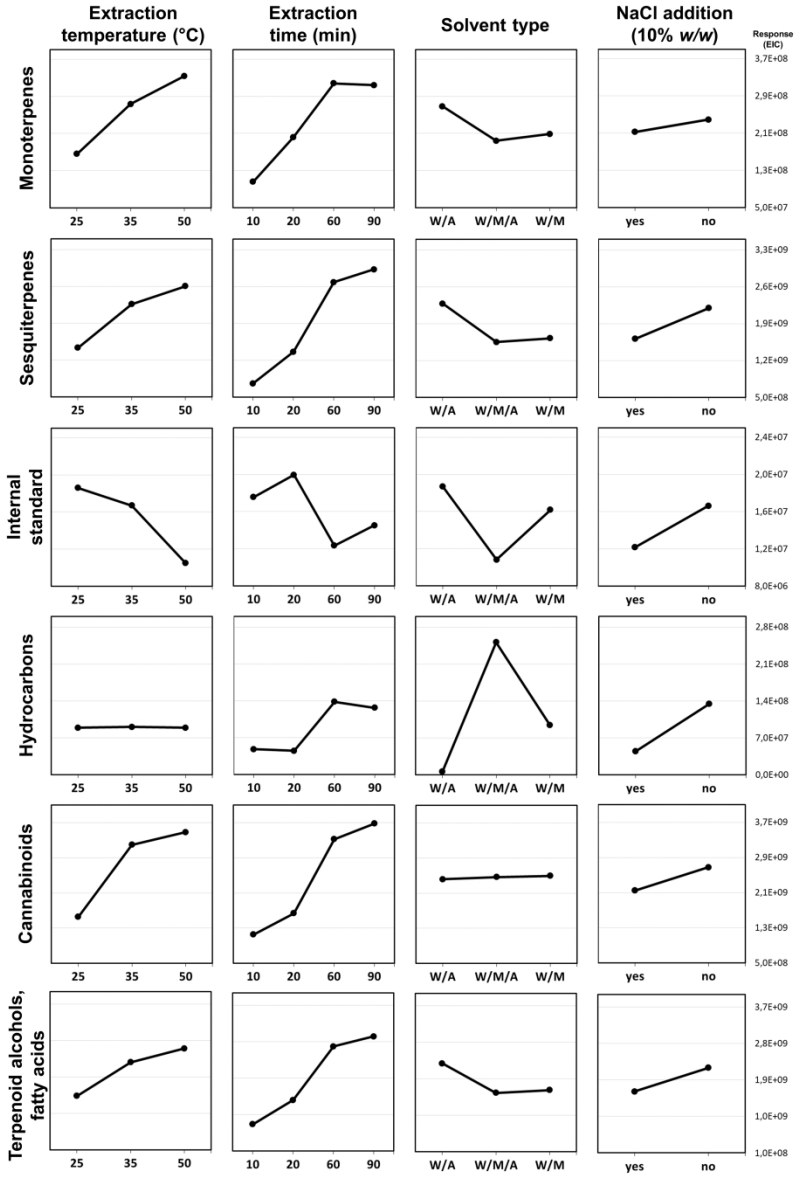

Figure 2. Main effect plot reporting the response under different extraction conditions (temp, time, solvent, salt addition) of mono-terpenes, sesquiterpenes, IS, hydrocarbons, cannabinoids, terpenoid alcohols and fatty acids. W = water, M = methanol, A = acetone.

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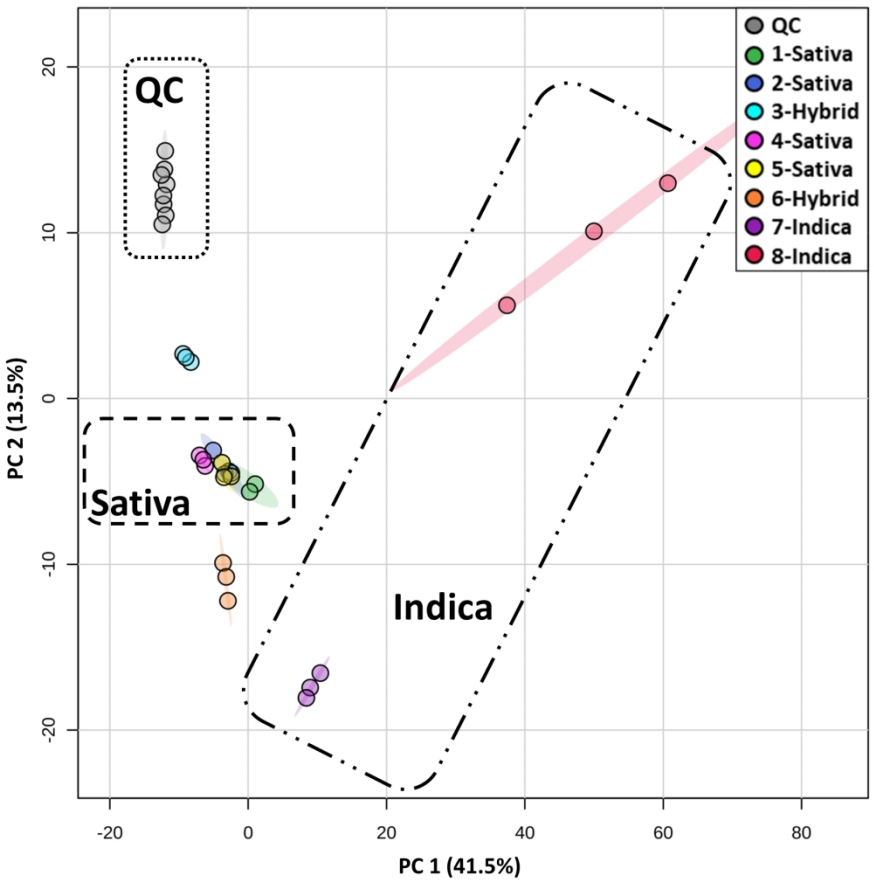


Figure 3. Untargeted PCA of the 754 features from the SBSE-GC×GC-MS untargeted analysis on cannabis inflorescences. Ellipses represent the 95% confidence interval for each group.

86x83mm (600 x 600 DPI)

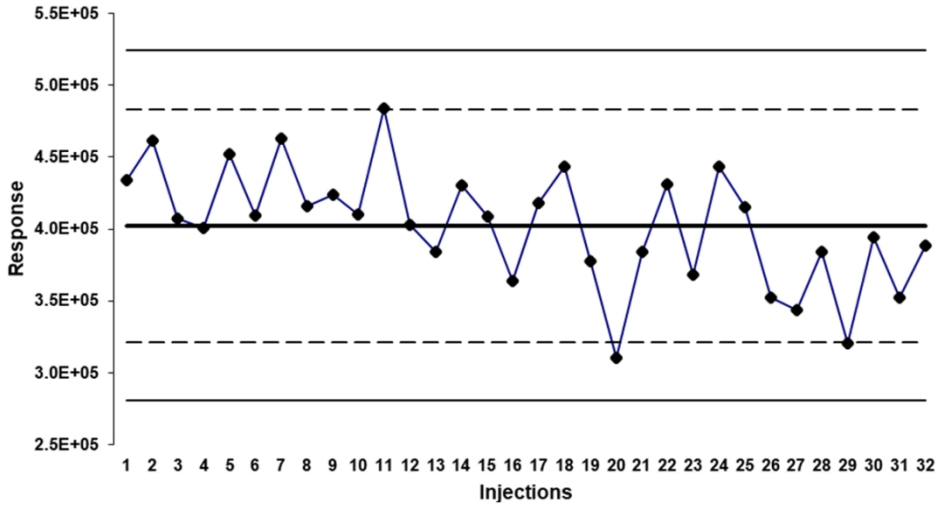


Figure 4. Control response chart of the internal standard in the cannabis and QC samples over time. The middle line represents the average value, the dotted lines represent the 2 σ and 3 σ lower and upper limits. A 14% RSD during the sample analyses was observed.

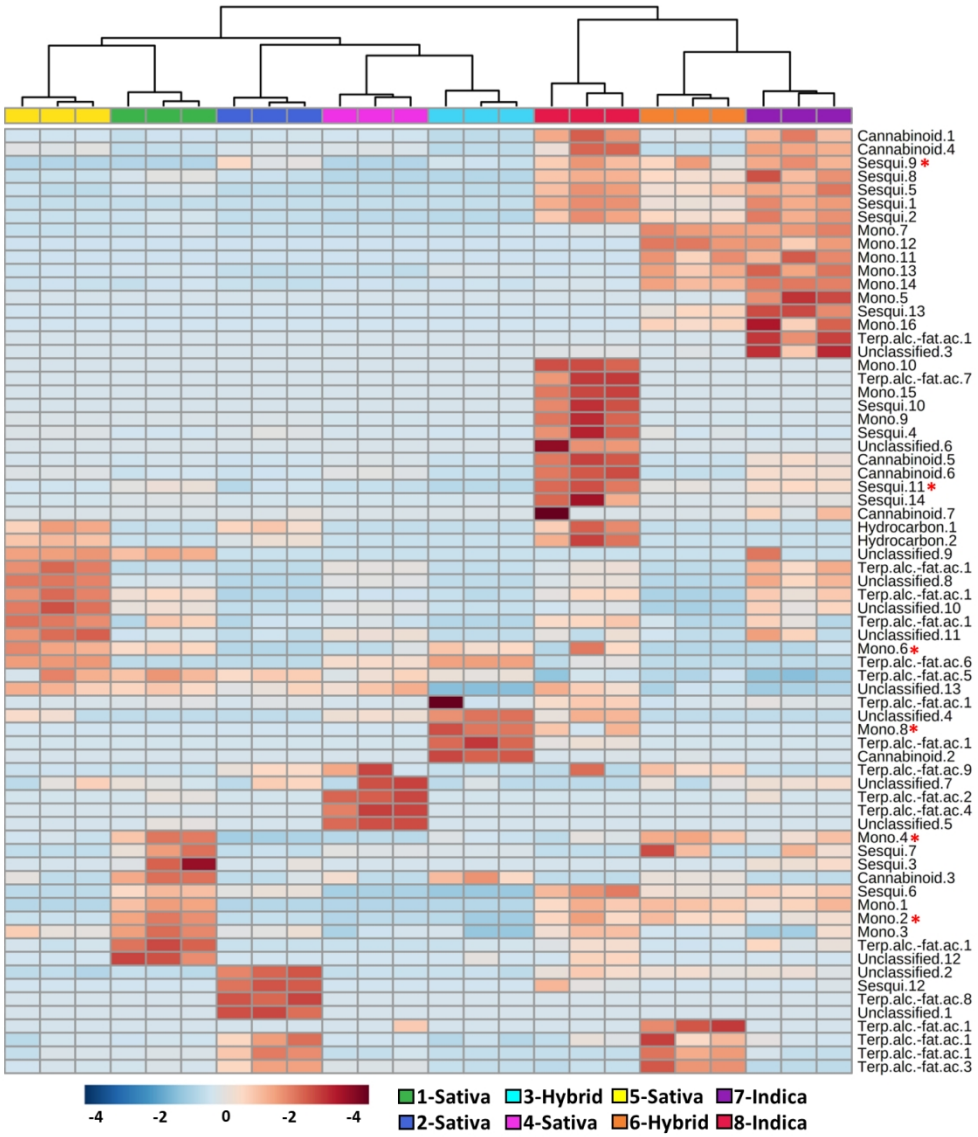
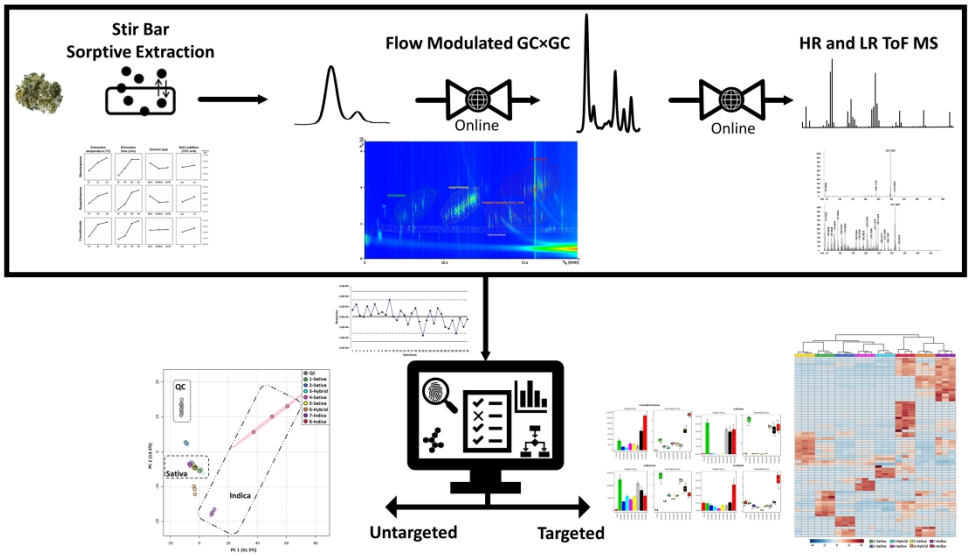


Figure 5. Heat map and hierarchical clustering analysis of 70 selected cannabis metabolites that were significantly different between the eight samples. The metabolites with * were confirmed via standard injection. The label "unclassified" represent the peaks eluting outside the classification regions in the GC×GC chromatogram.

67x77mm (600 x 600 DPI)



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141x79mm (600 x 600 DPI)