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Sorbent-Based Sampling With Two-Stage Trapping/Desorption Coupled to Comprehensive Two-Dimensional Gas Chromatography and Mass Spectrometry for Terpenoids Profiling in Cannabis

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ABSTRACT

Cannabis inflorescences represent an important source of many high-value bioactive specialized metabolites, among which the family of terpenes or terpenoids that are the largest classes of natural products known. Besides their biological activities either alone or synergistic with other terpenoids and/or cannabinoids, they are responsible for their distinctive flavour. In this study, we exploited the separation power and identification capabilities of comprehensive two-dimensional gas chromatography coupled to mass spectrometry (GC×GC-MS) for the profiling of terpenes and terpenoids in cannabis inflorescences. The dynamic headspace (DHS) used herein for the extraction was chosen for its sensitivity, portability, suitability, as well as its versatility of sampling various natural products, including plant raw materials and different plant parts. The enrichment method and the following desorption into the GC were developed and optimized on both standards and real samples considering different sorbent traps (i.e. Tenax-TA, Carbotrap T420, Carbotrap 202), and evaluating key performance values. Analyte coverage, recovery and response reproducibility were used for the evaluation of the best performing thermal desorption tube. Considering terpenoids profiling on cannabis inflorescences, satisfactory extraction performance was observed with both Tenax-TA and Carbotrap T420. However, Tenax-TA provided a wider analyte coverage beyond the class of terpenoids, thus can be better suited for non-targeted analysis. On the other hand, peak width, peak height, peak quality and resolution were considered for the optimization of the chromatographic process, and more specifically the injection process, demonstrating the benefit of a secondary trapping/desorption stage with a cryotrap. Finally, considering the final DHSE-TD-GC×GC-MS conditions, terpenes and terpenoids were profiled in real-world cannabis inflorescences, highlighting the differences among the chemovars.

1 | Introduction

Cannabis sativa L. (cannabis) is an extensively cultivated species belonging to the Cannabaceae family. Historically, in addition to representing a source of psychoactive substances, which makes it one of the world's most widely used recreational drugs, cannabis has been, and still is extensively used not only as a raw material for the textile and flavour industries [1, 2] but also in various applications as food products and medicinal formulations [3].

As with other natural products, many are the constituents of interest, both for the biological activity and the flavour. Terpenes or isoprenoids represent the largest class of secondary metabolites in cannabis and are mostly responsible for its characteristic aroma and exhibit therapeutic properties. These volatile molecules are involved in plant defence mechanisms as well as in plant-to-plant communication [4].

Chemically, this class of hydrocarbons derives from the fusion of several isoprene units of C_5H_8 and is classified based on the number of fundamental units, grouping them in monoterpenes (e.g. camphene, pinene and myrcene), sesquiterpenes (e.g. caryophyllene, humulene and bisabolene) and diterpenes (e.g. phytane and taxadiene) [5]. Terpenes can also be referred to as terpenoids when containing heteroatoms (i.e. oxygen).

A wide spectrum of biological activities can be attributed to the class of terpenes and terpenoids, among which the viricidal, antidepressant, antidiabetic, analgesic and antiplasmodial activities, attributing these properties to mainly beta-pinene, carvone, limonene, caryophyllene and linalool [6–9]. Even if not fully understood, these pharmacological effects depend on the synergistic interactions (also known as an entourage effect) with other terpenoids as well as cannabinoids [10, 11].

Studying and profiling terpenes and terpenoids in cannabis, as well as in other natural sources, could lead to the discovery of new therapeutic molecules, either directly or through the entourage effect. Furthermore, as a raw material for the medical, fragrance and food industries, chemical elucidation becomes important to verifying feedstock, identifying potential adulteration and ensuring product safety.

The determination of terpenoids in cannabis, as in other plants' raw materials, is commonly carried-out with one-dimensional gas chromatography (GC) with a flame ionization detector [12]. However, conventional GC methods often lack separation power for the full molecular separation of the sample constituents, especially when considering the complex composition of natural samples like cannabis and the variety of terpenoids known [13].

The use of comprehensive multidimensional GC (or GC×GC), thanks to its increased separation power, selectivity and sensitivity, can be considered as the technique of choice for a detailed molecular sample characterization. The increased separation power and sensitivity, among other advantages, usually provide an increased number of detected compounds from the complex mixture [14]. Noteworthy, such improved chromatographic resolution provides purer peak profiles reaching the detector. Therefore, when coupled to mass spectrometry (MS), high-quality mass spectra are acquired, resulting in a higher confidence for analyte identification.

Besides the separation technique, different extraction methods exist and the choice is of fundamental importance, and it depends on the sample type and objective(s) of the study [15–18]. In the case of cannabis floral parts, as for many other plant products, the use of headspace extraction (HS) is the logical and a preferred choice when the flavour profiling is sought. Opposite to liquid extraction, HS is based on solvent-free extractions, which can be exploited through static or dynamic mode and mediated by a solid sorbent or not, and the reader is directed to the literature for further information [19].

In this research, to directly profile the terpenes and terpenoids in cannabis inflorescences, we optimized and exploited a dynamic headspace (DHS) method using adsorption traps thermally desorbed into a GC×GC-MS system. Initially, we evaluated the efficiency in extracting/detecting the chemical groups of interest using probe standards, using traps packed with different adsorbents (i.e. Tenax-TA, Carbotrap T420 and Carbotrap 202), and applying a two-stage thermal desorption. Once we identified the optimal experimental conditions, we analysed the terpenoid profiles of commercial samples, highlighting the differences in the chemical composition and the benefits of using a multidimensional GC separation.

2 | Materials and Methods

2.1 | Chemicals, Standards and Samples

A test standard mixture of 12 compounds (Supelco, Bellefonte, PA, USA) was used initially for the evaluation of the cold trap as a secondary trapping/release stage. For the evaluation of the extraction performance of the different thermal desorption tubes (TDT), a mixture of 37 probe compounds was used (ID and GC×GC chromatograms reported in Figure 2).

Regarding the real-world samples, four CBD-dominant samples of *C. sativa* inflorescences were obtained from a local store (Ferrara, Italy) and transferred in a brown glass jar for storage at room temperature in dark conditions.

2.2 | Sampling and Extraction

DHS was used for both the standard mix and the cannabis inflorescences. Briefly, 2 µL of the probe mix was spiked into a 20 mL air-tight headspace glass vial and conditioned for 5 min at 40°C. A syringe was then used to manually draw 30 mL of room air through the vial headspace and onto the TDT at an approximate speed of 60 mL/min.

Regarding the samples, 20 mg of homogenized cannabis inflorescences were placed into a 20 mL air-tight headspace glass vial. Sample conditioning and dynamic extraction conditions were the same as for the standard mix. Three and four technical replicates, performed on separate TDT, were carried-out for the standard mix and the cannabis samples, respectively.

After each cycle of extraction/analysis, the tubes were conditioned using a Phoenix T220 (REDshift s.r.l., San Giorgio in Bosco, Padua, Italy) under the following conditions: 30°C/min to 320°C (held 60 min), under pure N_2 flow (100 mL/min). Tube blanks were conducted periodically to exclude carry-over.

Regarding the TDT, three different sorbent combinations, packed as single or multibed, were evaluated. Specifically, these were Tenax-TA (poly-(2,6-diphenyl-p-phenylenoxide)), Carbotrap T420 (Carbotrap F + Carbotrap Y) and Carbotrap 202 (Carbopack C + Carbopack B); for brevity, along the text and figures, these TDT are labelled as TA, CT420 and C202, respectively. All TDT were obtained from Supelco.

Initially, the extraction performance of each tube was investigated using the fragrance standard mix; then, only the tube types with higher extraction capacity and reproducibility were used to evaluate the terpenoids profile in real cases (i.e. cannabis inflorescences).

2.3 | Instrumental Experimental Conditions

The development of the GC×GC-MS method was conducted on a Pegasus BT 4D (LECO Corporation, Mönchengladbach, Germany) equipped with an Agilent 8890 GC and an automated tube handling device PAL System (CTC Analytics AG, Zwingen, Switzerland). The chromatographic columns were a 30 m × 0.25 mm × 0.25 µm d_f Rxi-5 ms as the first dimension and a 2 m × 0.25 mm × 0.25 µm d_f Rxi-17SilMS as the second dimension (both from Restek Corporation, Bellefonte, PA, USA). Injection was performed using an OPTIC-4 multimode injector (GL Sciences B.V., Eindhoven, Netherlands) equipped with an inlet Peltier cooler and a liner exchanger (GL Sciences B.V.). The GC was also equipped with a cryogenic trap (GL Sciences B.V.) as a secondary trapping/release stage. The carrier gas used was helium at 1.5 mL/min column flow throughout the run. The vent time (60 s) before thermal desorption consisted of split flow of 50 mL/min and a column flow of 0.5 mL/min, in which the inlet body was held at 20°C. During thermal desorption, the inlet was heated from 20° to 300°C at 20°C/s, using a 150 mL/min split flow and restoring the initial 1.5 mL/min column flow. The cryogenic trap was set at −20°C throughout the vent time (60 s) and the thermal desorption (45 s), after which its temperature was raised to 250°C at 50°C/s, allowing the secondary release of the analytes.

The oven temperature program was 40°C (held 1 min), then ramped at 4°C min^{-1} to 190°C, and finally ramped at 30°C/min^{-1} to 320°C (held 3 min). Temperature offsets for the secondary oven and for the modulator oven were set at +5°C and +15°C, respectively. A 3 s modulation period was used (cold and hot jets were 0.7 and 0.8 s, respectively). A time-of-flight mass analyser was used to acquire a mass range from *m/z* 40 to 500 at 150 Hz using electron ionization (70 eV). The ion source was maintained at 250°C, while the transfer line was set at 280°C. An acquisition delay of 180 s was used.

For the cryogenic trap evaluation, 2 µL of Grob mixture (10 ppm in hexane) was spiked on TA tube. Then, flushed at room temperature at 100 mL/min for 60 s. Chromatographic parameters were assessed under single- and dual-stage thermal desorption conditions in 1D GC.

Data were collected and analysed using ChromaTOF software version 5.56 (LECO Corporation), and NIST23 was used as a mass spectral library. The signal-to-noise threshold for peak integration was set at 10, and putative identification was based on the combination of spectral similarity (≥70%) and linear retention indices (RI) with a tolerance of ±30 units.

3 | Results and Discussion

3.1 | Optimization of Thermal Desorption and Sorbent Selection

3.1.1 | Advantages of Secondary Trapping/Release Stage

The instrumental configuration for thermal desorption involved the use of a multimode injector with an automated tube exchanger. Such a configuration avoids the use of an external unit, making the system more compact and with fewer hardware components, providing a direct and more efficient injection into the GC column head. Nevertheless, the overall chromatography can benefit of an additional trapping/release stage involving the use of a cryotrap. Therefore, a first step of the method development regarded the evaluation of the cryotrap conditions.

For such a purpose, a test standard mixture (i.e. Grob mix) was used, and full width at half height, tailing factor, peak height and resolution were evaluated with ('trap active') and without the trap ('trap not active'). During thermal desorption indeed, analytes ideally are transferred from the tube into the GC column as narrow bands, and the presence of an additional focusing system (dual-stage thermal desorption) reduces both band broadening and breakthrough of highly volatile compounds for a better final separation [20]. Not surprisingly, the secondary trapping/release stage reduced the average full width at half-height (FWHM) from 2.9 to 2.1 s, while the tailing factor dropped from an average of 1.6–1.5 and an average increase of 28% in peak intensity was also observed.

Figure 1 shows the overlapped 1D chromatogram with and without the trap, in which the chromatographic improvement can be observed in terms of peak shape, peak height and resolution between some critical pairs. The use of the secondary trapping/release stage increased considerably the chromatographic resolution between the undecane/nonanal and 2,6-dimethylphenol/2-ethyl-hexanoic acid by a factor × 1.7 and × 2.5, respectively. Such advantages observed in one-dimensional chromatography are beneficial also in two-dimensional separations.

3.1.2 | Adsorbents Performance for DHS

Before sampling from the real case of cannabis inflorescences, we evaluated the extraction efficiencies of three differently packed TDT on probe analytes. Such a probe mixture was selected to match the range and class of compounds expected in the cannabis inflorescences, covering different degrees of analytes' polarity and boiling point. In addition to terpenes (mono- and sesqui-),

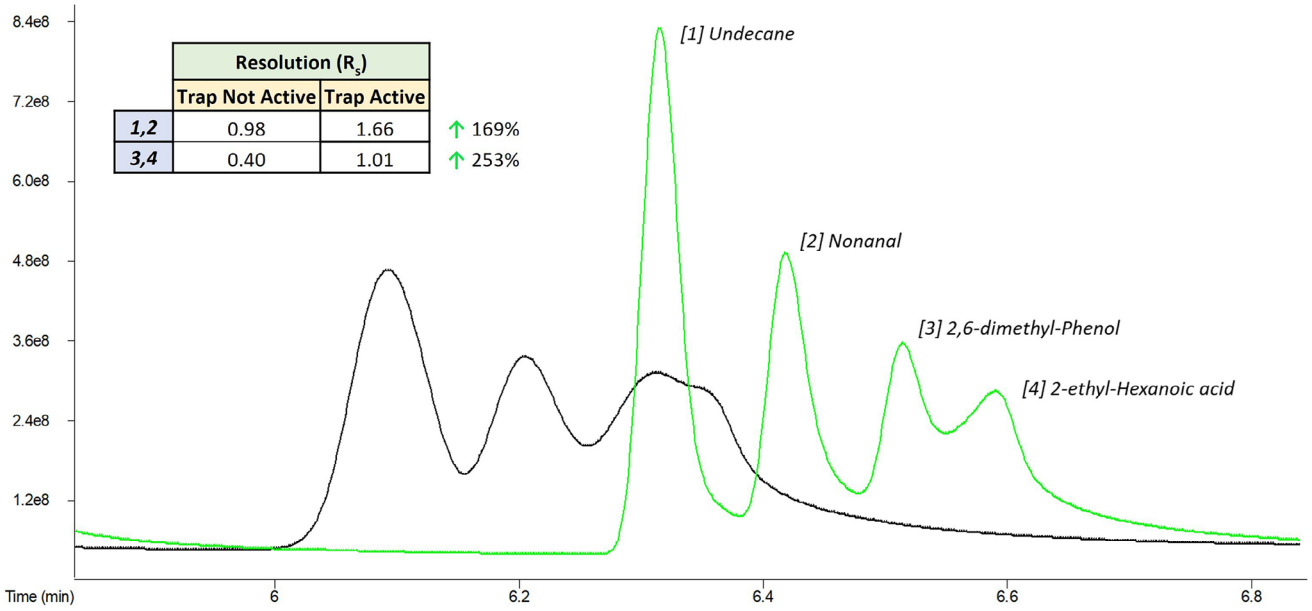


FIGURE 1 | Zoomed GC chromatogram of critical pairs obtained with single-stage (black trace) and dual stage-thermal desorption (green trace).

it contained other aldehydes, ketones and alcohols (composition listed in Figure 2), with various polarities and boiling points. Dynamic extraction was performed keeping the same conditions (see Materials and Methods) and by varying the TDT types. The separation of the 37 analytes resulting from the optimized TD-GC×GC conditions is shown in Figure 2A.

The response observed with the different tube types is variable, and it is related to the properties, thus the selectivity of the adsorbent materials [21–23].

In terms of analyte signals, C202 tubes showed substantially lower signals compared to TA and CT420, thus lower extraction efficiency (Figure 2B). Even though TA tubes highlighted an overall better response, the CT420 tubes were comparable on some terpenes (e.g. β-myrcene, o-cymene, eucalyptol and limonene). It is also worth noting that the extraction performance of the TA compared to the CT420 was 33.2% higher, considering the average value of the 37 probe analytes. Therefore, TA and CT420 tubes were further used for the investigation of real-world samples.

3.2 | Volatile Organic Compounds Profiling of Different Cannabis Chemovars

The volatile profile of four different cannabis chemovar inflorescences was extracted using TA and CT420 tubes, thanks to their satisfactory extraction performance proven with the probe mixture and discussed in the previous paragraph.

Figure 3A shows a representative 2D chromatogram obtained with TA tubes. The primary non-polar and secondary polar column combination allows for the clustering of the two main terpene's classes, that is, monoterpenes and sesquiterpenes. For the rapid classification of these terpenoids, two elution regions were drawn based on the RI information (considering both experimental and literature values) and the characteristic fragments (e.g. m/z 69, 93, 105, 121 and 136). These two elution regions are circled and spanned from an RI of 877 (i.e. the monoterpene cyclofenchene) to 1810 (i.e. the sesquiterpenoid nootkatone [24]).

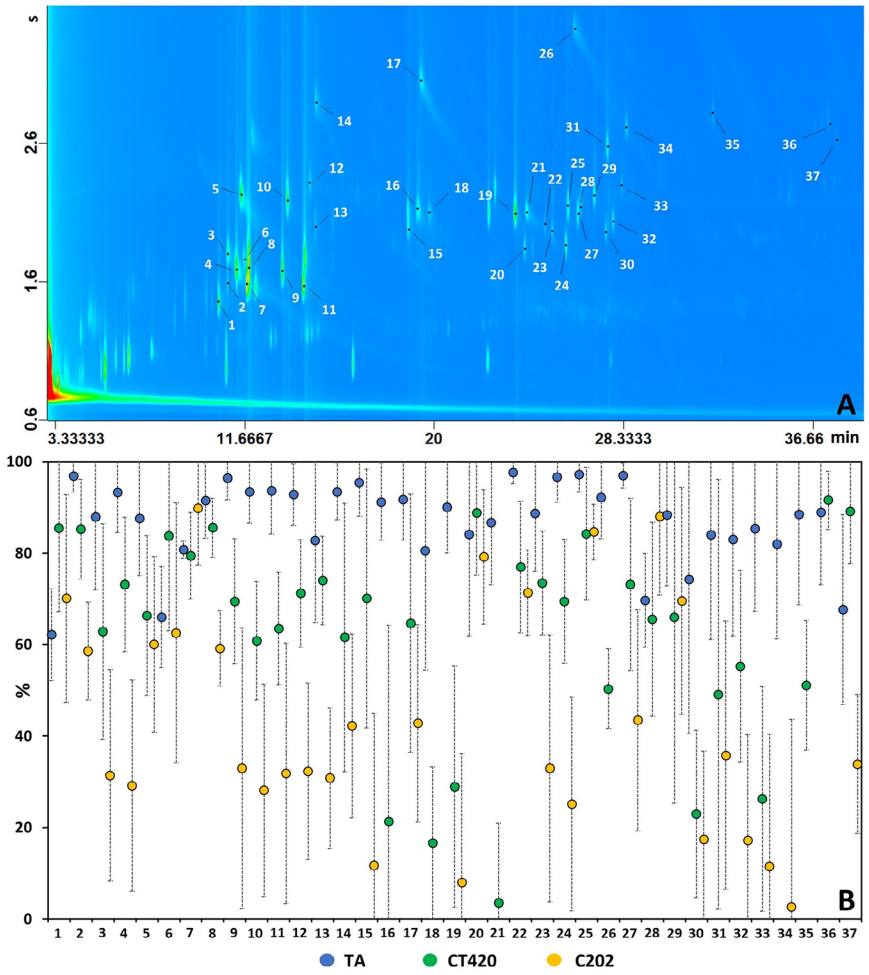
Considering the large number of compounds present in cannabis inflorescences, GC×GC certainly proves to be a suitable separation technique. Some examples of peak pairs that would be otherwise coeluted in conventional 1D separation are shown in Figure 3B,C. In the case of the monoterpenes terpinolene and fenchone (Figure 3B), the resulting chromatographic resolution improved from 0.24 to 1.51, respectively, in 1D and 2D. Similarly, the sesquiterpenes 3,7(11)-selinadiene and α-bisabolene gained a resolution of 1.14 using GC×GC, on the contrary to 0.19 in 1D (Figure 3B). One more benefit arising from the improved GC×GC separation is visible in Figure 3B,C and regards the purity of mass spectra, which resulted in a higher spectral match with reference databases.

On average, considering the 2 terpenoid elution regions, 183 analytes were detected in the sample headspace and using TA.

In order to compare the performance of the two selected TDTs, 75 putatively identified terpenes were considered from 4 different cannabis chemovars; these are listed in Table S1 and visually highlighted in Figure S1. The identification criteria and the information on the samples are reported in Materials and Methods section.

Of the 75 putatively identified compounds, 43 were classified as monoterpenes and 32 as sesquiterpenes, based on the elution region.

It must be highlighted that additional compounds were detected, eluting within these regions (e.g. on sample #1, 45 compounds within the monoterpene and 45 compounds within the sesquiterpenes region), and are marked with red and green dots on the



Peak	ID	Characteristic *m/z*
1	β-Myrcene	93
2	Pseudolimonen	93
3	Octanal	43
4	Acetic acid, hexyl ester	43
5	Methyl p-cresyl ether	122
6	o-Cymene	119
7	D-Limonene	68
8	Eucalyptol	43
9	Dihydromyrcenol	59
10	Cyclal C	67
11	Tetrahydro linalool	73
12	Tricyclal	67
13	Fenchol	81
14	Phenylethyl Alcohol	91
15	Citronellol	69
16	Allyl (3-methylbutoxy)acetate	43
17	Methyl phenethyl ketone	43
18	Nerol	69
19	Vertenex	57
20	β-Patchoulene	161
21	Lavandulyl acetate	69
22	Cycloseychellene	123
23	Caryophyllene	93
24	α-Guaiene	105
25	Seychellene	122
26	Yara yara	115
27	α-Patchoulene	93
28	γ-Patchoulene	121
29	α Isomethyl ionone	135
30	β-Bisabolene	69
31	Cashmeran	191
32	α-Bulnesene	93
33	α-N-Methyl ionone	121
34	Lilial	189
35	α-pentyl-Cinnamaldehyde	91
36	Versalide	243
37	Tonalid	243

FIGURE 2 | GC×GC plot (TIC) of probe fragrance mixture using TA as trapping material (A). Analyte response and reproducibility (error bars, $n = 3$) with thermal desorption tubes (blue dots = TA; green dots = CT420; yellow dots = C202) (B); Y-scale is normalized for each analyte to the highest signal among the three tube types. For peak numbering, please refer to peak list on the right.

2D plot in Figure S1. Considering the spectral fragmentation patterns, these can be reasonably other terpenoids, which were not included in the comparison because of a lower identification level (SI < 700 and ΔRI > 30 or absent).

Figure 4 shows the performance of the tubes regarding the terpenoids in the four different chemovars in terms of total response and number of detected peaks. On the one hand, this response gives an indication of the extraction efficiency of the trapping materials; on the other hand, the number of detected peaks highlights the selectivity and analyte coverage of trapping materials towards terpenes.

Besides the different distribution of monoterpenes and sesquiterpenes among chemovars, a consistent trend of the extraction performance can be highlighted.

Focusing on the difference between TA and CT420 in Figure 4A, the former slightly outperforms in terms of signal, and more importantly, in terms of reproducibility (on average 5.4 and 7.5 for monoterpenes and sesquiterpenes for TA vs. 7.9 and 9.7, respectively, for CT420).

Another observation can be highlighted from Figure 4B: here, the number of detected peaks (S/N > 10) is reported with the two different extraction TD tubes. Tubes packed with Tenax extracted more peaks than CT420, confirming a higher universality and broader selectivity. A detailed distribution of the 75 terpenoids among the 4 chemovars is shown in the heatmap of Figure S2. In addition to observing the differences among the four chemovars at the single analyte level, it is possible to compare again the varying extraction performance according to the packing material.

Non-targeted analysis, different from profiling of specific chemical classes, has the objective to gather and retain as much information from the chemical composition, limiting bias and manipulation of the sample [25]. Even if a mere non-targeted approach is outside the scope of the current research, some observations and considerations are still relevant on the use of the sorbent materials herein tested. The broader selectivity of TA tubes discussed earlier was also observed with the detection of compounds, which were not extracted by CT420 tubes. An example of compounds uniquely extracted and detected when using TA is shown in Figure S3. These two compounds elute

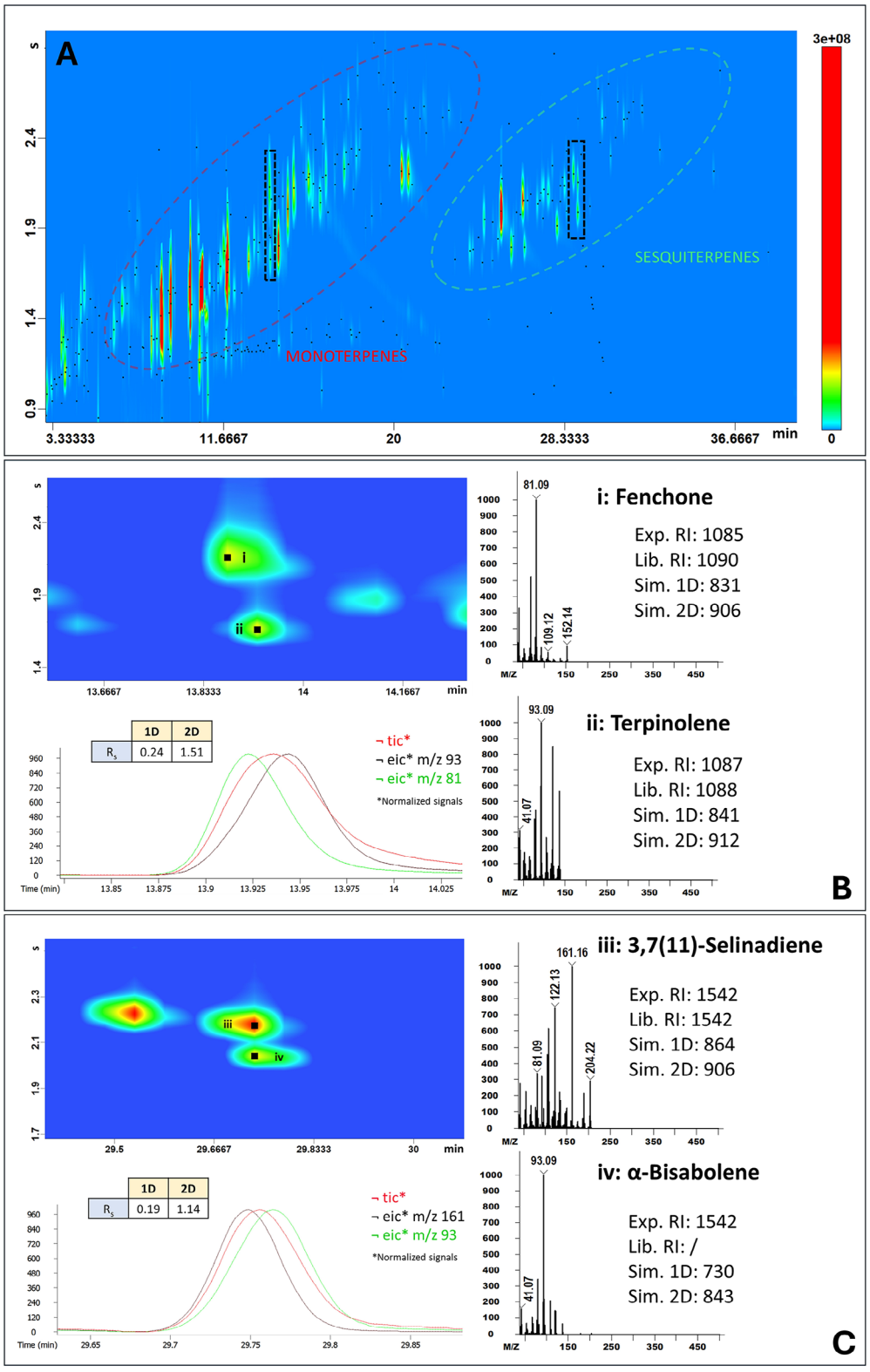


FIGURE 3 | 2D elution regions of terpene classes (A). Chromatographic resolution comparison of selected terpenes between 2D and 1D, with relative spectral and RI information (B and C).

in between the monoterpene and sesquiterpene regions are not present in the blanks, and they do not meet a reliable identification criterion (SI > 700 and ΔRI < 30). The mass spectra of these analytes, probably not contained in the MS database, are also reported in Figure S3.

4 | Conclusions

This study revealed how the use of DHS followed by thermal desorption represents an effective and non-invasive approach for the investigation of volatile profiles of cannabis inflorescences,

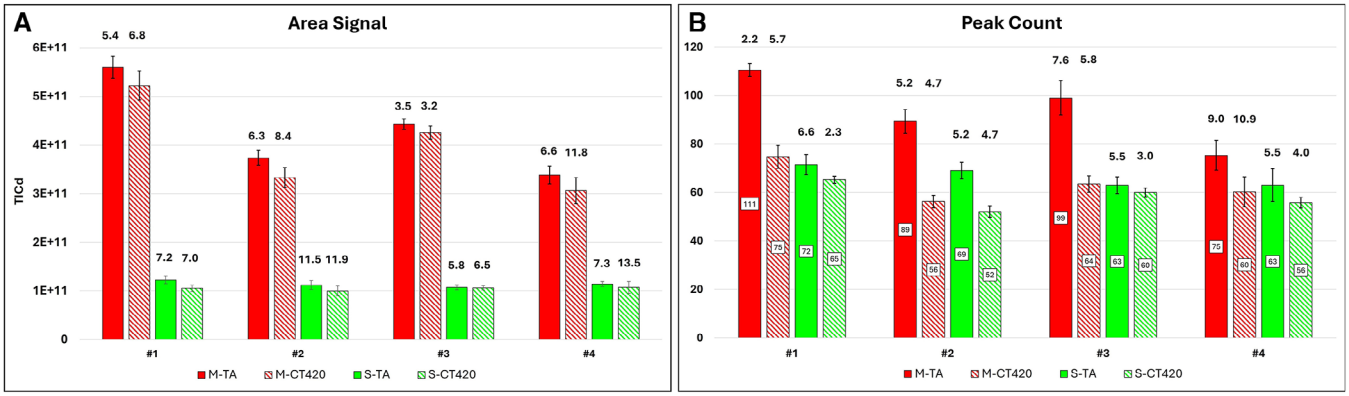


FIGURE 4 | Total signal area comparison on monoterpenoids and sesquiterpenoids between Tenax-TA and Carbotrap T420 (A). Tube collection efficiency on peak count (B). Error bars represent the standard deviation ($n = 4$), which is also reported numerically on top of each bar.

which can be advantageous for *on-field* applications. Using both probe standard analytes and real-world samples, we showed that the secondary trapping/release stage herein implemented greatly boosts the chromatographic performance (i.e. peak quality and resolution).

For the choice of the sampling adsorbent material, we observed that either the Tenax-TA (porous polymer) or the Carbotrap T420 (combination of two graphitized carbon blacks) tubes are suitable for terpenoids profiling in plants, even though the use of the former one provides better reproducibility. However, when the scope of the investigation goes beyond a targeted or a profiling approach, the use of the porous polymer grants an extended coverage of analytes, and thus it is more suitable for non-targeted purposes.

Regarding the separation technique, we showed that the use of comprehensive 2D GC coupled to MS allows a deep molecular characterization of cannabis with enhanced resolution and identification reliability, highlighting flavour differences based on the chemovars.

Author Contributions

Marco De Poli: data curation, formal analysis, investigation, methodology, validation, visualization, writing–original draft. **Tatiana Chenet**: writing–review and editing. **Simona Felletti**: writing–review and editing. **Damiana Spadafora**: writing–review and editing. **Alberto Cavazzini**: resources, writing–review and editing. **Flavio A. Franchina**: conceptualization, funding acquisition, methodology, project administration, resources, supervision, validation, visualization, writing–original draft, writing–review and editing.

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[Correction added on 23 December, 2024 after first online publication: CRUI-CARE funding statement has been updated in this version.]

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data are available on request from the authors.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.