A Systematic Approach to Developing Terpene Extraction Conditions Utilising Supercritical Carbon Dioxide

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Cannabis Sativa plants produce and accumulate terpene-rich resin within the secretory cells of glandular trichromes [1]. Monoterpenes and sesquiterpenes are important components of Cannabis resin as they contribute to the unique attributes of different Cannabis strains. Terpenes are responsible for the plant's aroma and flavour.

They possess specific medical properties and may act synergistically with cannabinoids, enhancing the therapeutic benefits of the plant. The extraction of terpenes and cannabinoids from Cannabis is a function of their solubility in different organic solvents [2]. Solvents like methanol, ethanol, butane, and hexane are commonly used in Cannabis extractions. However, aside from safety considerations, extracts produced with such solvents are considered 'one pot extractions'; no selectivity between cannabinoids and terpenes can be achieved.

Discussing terpene extraction is problematic for two reasons.

1. during post-processing, thermally labile terpenes undergo degradation reactions
2. isolation of terpenes from these solvents is difficult due to similar boiling points

Among the various extraction techniques, we explore the use of supercritical fluids (SC) as a solvent for the targeted extraction of terpenes from cannabinoids in Cannabis.

Data suggests that the interaction between cannabinoids and terpenes affects the pharmacological properties of cannabis strains; this relationship is commonly referred to as the 'entourage effect' [3]. There are several promising applications based on the combined use of cannabinoids and terpenes, such as pairing cannabidiol (CBD) with the monoterpenes limonene, linalool and pinene to treat acne [4] or adding caryophyllene, linalool and myrcene to 1:1 CBD/THC extracts to treat sleeping disorders [4].

The smallest and most volatile terpenes are monoterpenes which are biosynthesised by the head-to-tail addition of two isoprene

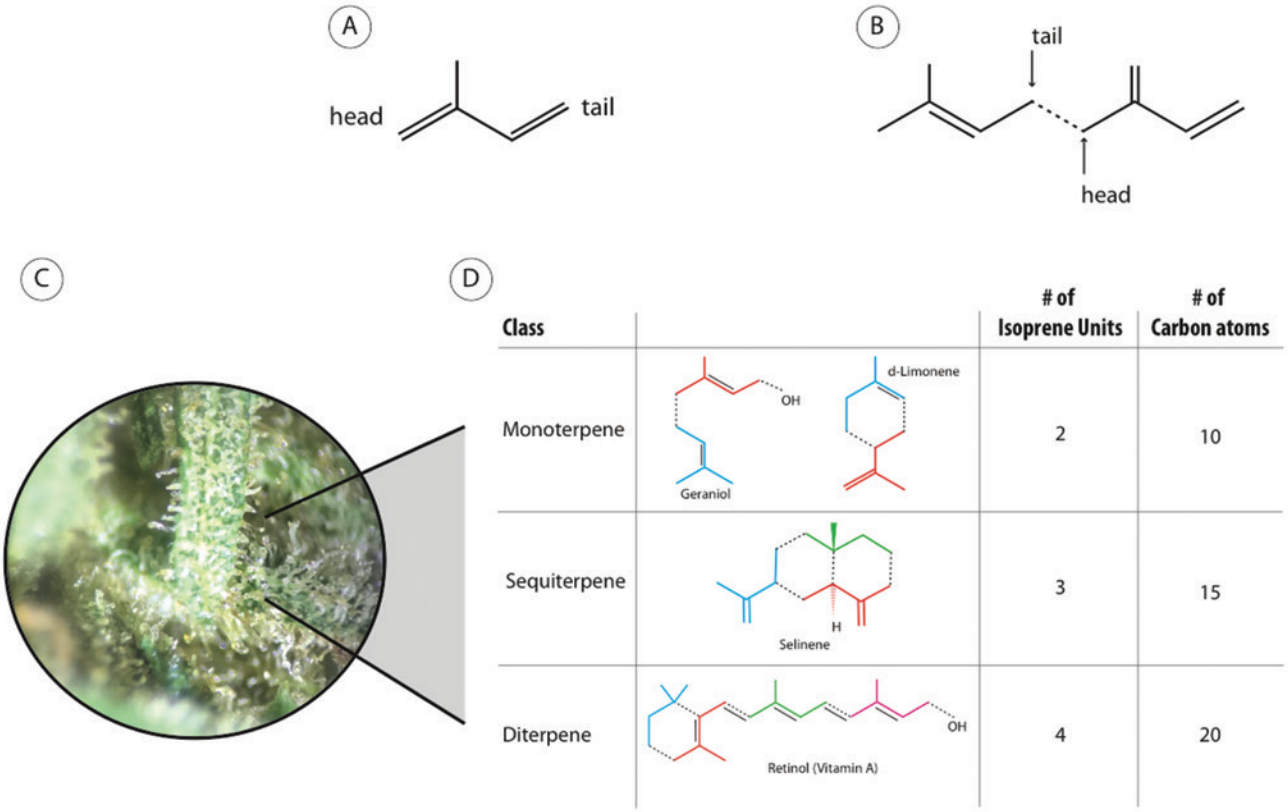


Figure 1: (A) Five carbon isoprene unit (B) head to tail linkage of two isoprene units to form the monoterpene Myrcene (C) terpene-rich resin within the secretory cells of glandular trichromes (D) classification of terpenes based on the number of isoprene units.

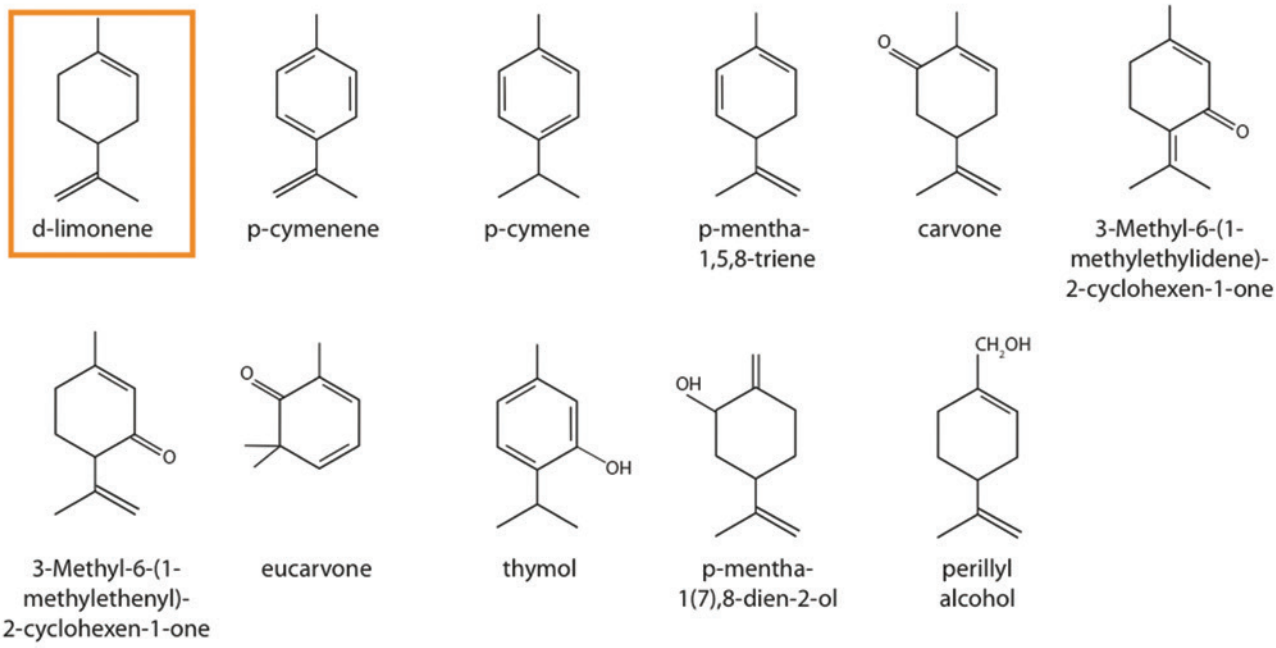


Figure 2: Products of the thermal degradation of the monoterpene limonene when heated at 120°C for 24 hours. Degradation of Limonene was monitored by GC-FID. 50% of Limonene degraded in 24 Hours into the compounds depicted above [5].

units (Figure 1). An isoprene unit is a 5-carbon molecule upon which terpenes are built. Combining three isoprene units forms a class of compounds called sesquiterpenes which are less volatile than monoterpenes. The largest and least volatile terpenes are biosynthesised by the joining of four or more isoprene units.

Terpenes are both volatile and thermolabile compounds. They easily convert into each other by oxidation, isomerisation, cyclisation or dehydrogenation reactions (Figure 2). The environment in which Cannabis is stored and the methods used in processing will effect the chemical composition of these isoprene-containing compounds. For example, distillation of crude Cannabis oil raises two issues. First, due to the high temperature required for distillation, delicate monoterpenes readily thermally degrade. Second, during distillation organic solvents co-elute with the terpene fraction. If this terpene fraction is used in Cannabis oil formulation, these organic solvents will contaminate the final product.

Supercritical fluid extraction (SFE) is an effective method for the separation of monoterpenes from sesquiterpenes, their alcohol derivatives, and cannabinoids. The most popular supercritical solvent used is carbon dioxide (CO_2). CO_2 is inexpensive and is a generally recognised as safe solvent. CO_2 reaches supercritical state at conditions of 31°C and 74 bar and returns to a gaseous state at ambient conditions. This allows for simple solute recovery and results in a solvent-free extract [6]. By modifying the pressure and temperature of the CO_2 system, the dissolution properties of the solvent can be adjusted, enabling a selective extraction of cannabinoids and terpenes. Here we describe a systematic approach to developing terpene extraction conditions utilising supercritical carbon dioxide

Experiment 1

In order to develop terpene extraction conditions it is critical to understand the relative solubility of terpenes in supercritical carbon dioxide. Solubility data exists for cannabinoids in supercritical carbon dioxide [[2], but limited literature is found on the solubility of terpenes. In Experiment 1, we increase our understanding of the elution order of monoterpenes and their separation from sesquiterpenes at different supercritical carbon dioxide conditions.

To develop and optimise conditions for terpene extraction using supercritical carbon dioxide, we used the Investigator Supercritical Fluid Chromatography (SFC) instrument (Waters, Milford, USA). Typically, the Investigator SFC system is used for analytical and semi-preparative purification work [7]. However, with a few

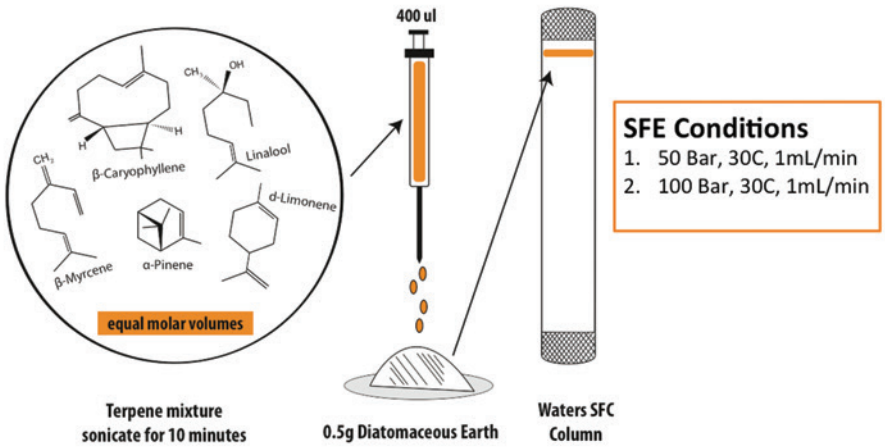
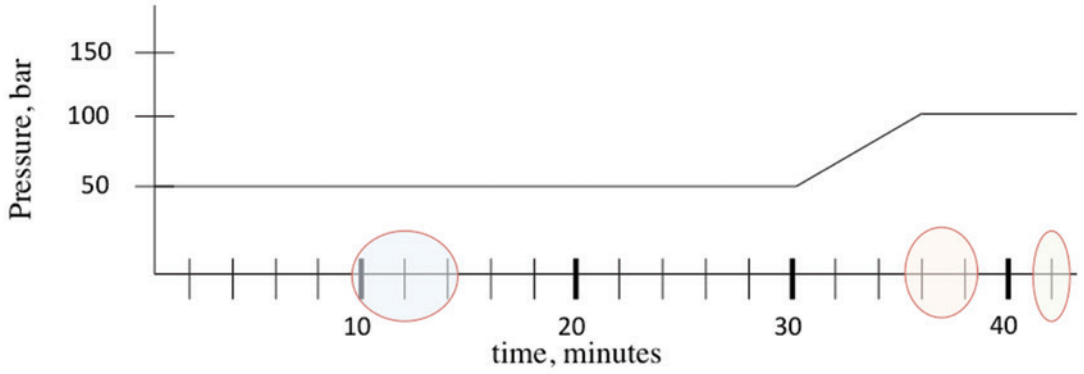


Figure 3: Investigator SFC System/experimental design.

Table 1: Relative solubility of α-Pinene, β-Myrcene, d-Limonene, Linalool, β-Caryophyllene. The modified SFC Investigator system was equilibrated and SFE parameters maintained at 50 bar, 30°C with a flow rate of 1ml/min for 30 minutes after which the density of CO_2 was changed by increasing the pressure to 100 bar. Fractions were coloured coded to indicated time of elution.



	Starting Material	F#1	F#2	F#3	F#4	F#12	F#18	F#24
α–Pinene	0.65	0.38	**0.78**	0.48	0.4	/	/	/
β -Myrcene	0.52	0.39	0.39	**0.73**	0.6	0.04	0.03	/
d-Limonene	0.78	0.65	0.65	**0.87**	0.77	0.07	0.09	/
Linalool	1	1	1	1	1	1	1	1
β -Caryophyllene	0.83	0.77	0.84	0.85	0.84	**7.38**	2.64	1.1

minor modifications, this SFC unit can be transformed into a bench top preparative SFE system.

First, a 20 mL SFC column was obtained and the column packing was removed, effectively transforming it into an extraction vessel for the bench top SFE experiments.

Next, a three-way valve was installed before the photodiode array detector and 1/16 inch tubing was plumbed directly into the backpressure regulator, bypassing the detector. The Fraction Collection Module was disconnected and fractions, already enriched with the target compounds, were collected as material exited the automatic backpressure regulator (ABPR) into scintillation vials.

The SFC column was pre-packed with diatomaceous earth. Equal molar amounts of α-pinene, β-myrcene, d-limonene, linalool, β-caryophyllene were sonicated for 10 minutes. 400 µL of this solution was injected onto 0.5 g of diatomaceous earth and loaded into the head of the 20 mL pre-packed SFC column. The column was installed into the Investigator's oven, and the system was equilibrated to 50 bar with the oven temperature set to 30°C. A flow rate of 1 mL/min was maintained throughout the extraction process.

From a starting mixture of 400 µL of terpines a total of twenty-four 15 µL fractions were collected in 42 minutes employing pure CO_2 as mobile phase. The elution of terpenes began at 10 minutes. Fractions 1 through 11 were collected between 10 and 14 minutes after which the elution of terpenes stopped. At minute 30, the system was equilibrated to 100 bar, maintaining the initial column temperature and flow settings. Terpene elution was once again observed at minute 36. Fractions 12 – 24 were collected between minute 36 and 42. Each fraction was analysed using GC-FID and the peak height recorded. Linalool and beta-caryophyllene were observed in each fraction. Linalool was chosen as the normalisation peak as it produced the highest signal. To standardise the data, the peak height of each terpene was divided by the peak height of linalool.

In Table 1, the terpene fractions with the highest terpene:linalool ratio are highlighted in bold. By comparing these ratios, we can determine the elution of terpenes at given supercritical carbon dioxide conditions. Alpha-pinene was found at its highest concentration in fraction 2, followed by beta-myrcene and d-limonene. These monoterpenes all eluted at 50 bar and 30°C. A ratio turning point for beta-caryophyllene occurred in fraction 12; the ratio is above 1, meaning there was a higher concentration of beta-caryophyllene than linalool. This suggests that the solubility of these terpenes at the given supercritical carbon dioxide conditions is as follows: α-pinene, β-myrcene, d-limonene, linalool, β-caryophylene.

Experiment 2

Next, the conditions from Experiment 1 were used to extract 5 grams of Cannabis inflorescence (the mature flower of a female plant). A similar procedure was followed: this time a 5 mL SFC column was obtained and the packing removed. Five grams of cannabis inflorescence were ground, packed into the 5mL SFC column, and installed into the Investigator's oven. The Investigator system was equilibrated to 50 bar and the oven set to 30°C. The flow rate was maintained at 7 mL/min. No fractions were observed in the first 60 minutes, at which time the system was equilibrated to 100 bar, maintaining temperature and flow rate. Three fractions were collected, with the elution starting at 80 minutes. These fractions were analysed using GC-FID and compared with the terpene quantification of the starting material.

Results were not satisfactory for two reasons:

1. In this first attempt we were unable to recover our volatile terpenes
2. The length of extraction was prohibitively long

If these conditions were scaled to a 5 L system (vs our 5 mL system), the terpene extraction would take over three hours, requiring a significant amount of CO_2. This experiment was repeated multiple times, modifying both the instrumentation and supercritical carbon dioxide conditions until both monoterpenes and sesquiterpenes were recovered in an appropriate length of time. The next section will examine the modifications and conditions required to scale up to a 5 litre SFE system.

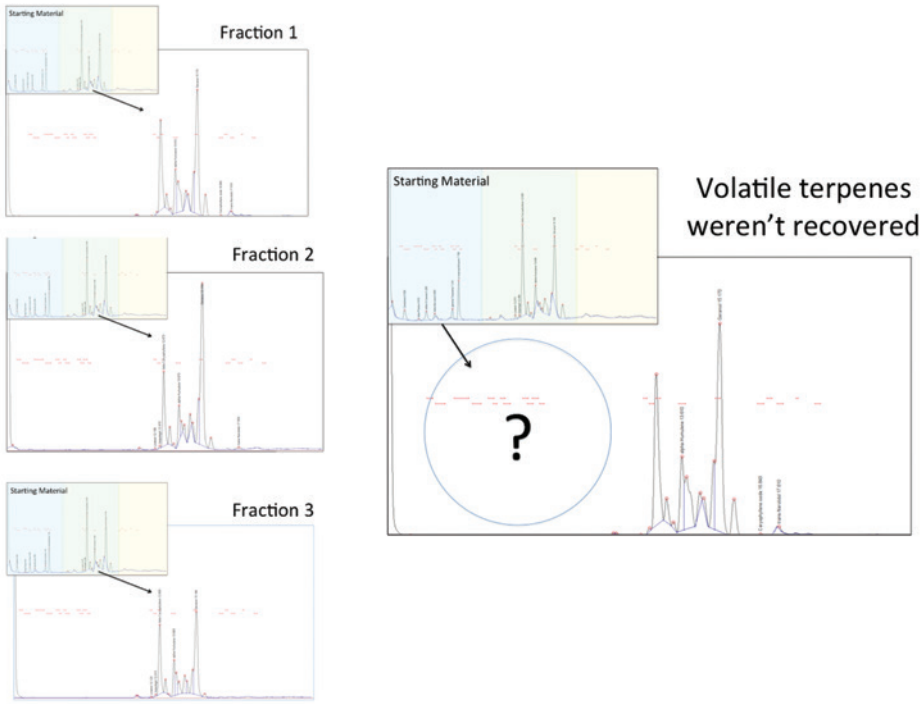


Figure 4: GC-FID analysis of the three terpene fractions obtained from the 5 g Cannabis extraction

Supercritical Extraction Equipment and Terpene Extraction Procedure

The scale up terpene extraction was conducted using a 5 L Bio-Botanical Extraction System (Waters, Milford, USA) modified to include a 4th terpene collection vessel (CS4). The system had a maximum operating pressure of 600 bar. Extraction pressure was maintained by the ABPR. An electrical jacket controlled the temperature of the extraction vessel. The system had three collection vessels (CS1-CS3), each independently heated and each with manual pressure control. The high-pressure pump delivered a maximum mass flow of 200 g/min. The solvent flowed through a heat

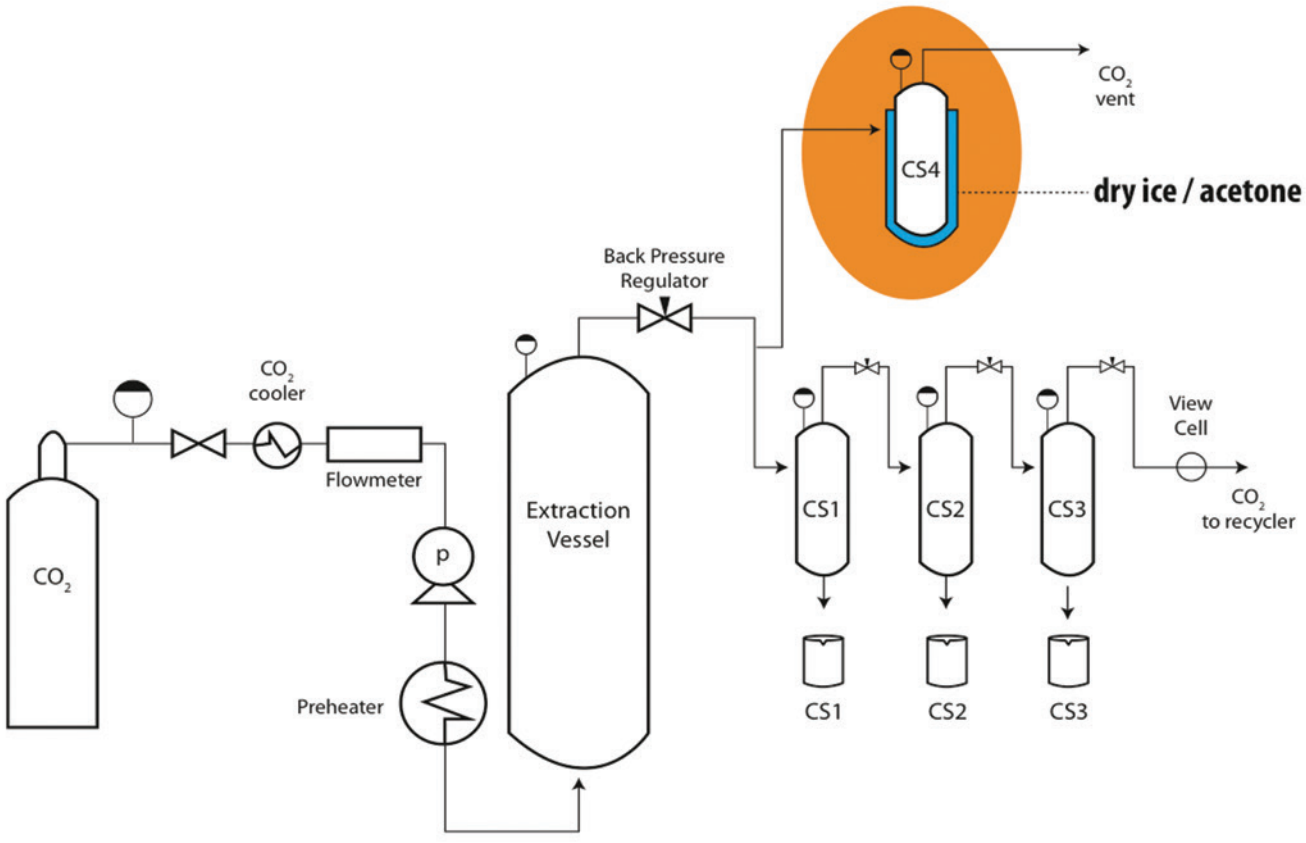


Figure 5: Schematic drawing of 5 litre Bio-botanical Extractor (Waters, Milford, USA) modified with an extra 4-terpene cyclone collection vessel. (CS = cyclone separator)

Table 2: Optimised Supercritical CO_2 extraction conditions for targeting terpenes found in terpene rich Hemp.

	Stage 1	Stage 2	Stage 3
Extraction Temperature (°C)	50	55	55
Extraction Pressure (bar)	70	150	300
Flow Rate (g/min)	100	150	200
Extraction time (min)	45	45	45
CS1 Pressure (bar) / Temperature (°C)	N/A	103 / 50	103 / 50
CS2 Pressure (bar) / Temperature (°C)	N/A	65 / 45	65 / 45
CS3 Pressure (bar) / Temperature (°C)	N/A	51 / 35	51 / 35
SC4 Pressure (bar) / Temperature (°C)	Ambient / -78	N/A	N/A

Extraction Time Terpenes	*45 min*
Extraction Time Cannabinoids	*5.5 hours*
Extraction Efficiency	*92%*

exchanger to bring the liquid CO_2 to a supercritical state before it entered into the extractor vessel. The supercritical stream dissolved the target components from the botanical material and directed them from the extraction vessel to the appropriate cyclone separators. The optimised conditions are summarised in Table 2.

Cannabinoids and terpenes have varying solubility in supercritical carbon dioxide. Furthermore, compounds within the classes of cannabinoids and terpenes have varying solubility in supercritical carbon dioxide. The extraction of terpene rich hemp is divided into three stages, with the density profile of supercritical carbon dioxide being increased at each stage in order to target different compounds.

To extract monoterpenes, the flow of supercritical carbon dioxide was first directed into CS4, bypassing cyclone separators one through three. Without the addition of CS4, volatile monoterpenes evacuate with supercritical carbon dioxidefrom SC3 into the CO_2 recycler. With the addition of CS4, it is possible to create conditions that allow for the recovery of volatile monoterpenes. However, even with the addition of CS4, at ambient temperatures volatile monoterpenes will evacuate with the gaseous CO_2. To remedy this, CS4 was cooled to -78°C with a solvent mixture of acetone and dry ice. Once chilled, the extraction vessel was pressurised to 70 bar and extraction occurred at 50°C. Monoterpenes have high solubility in supercritical carbon dioxide at these conditions, while sesquiterpenes have mild to low solubility. In Stage 2, both temperature and pressure were increased to target less volatile sesquiterpenes. During this stage, the flow of supercritical carbon dioxide was directed into CS1 and continued through CS3. After 45 minutes, sesquiterpenes were collected from CS3.

Both terpene fractions were analysed using GC-FID, shown in Figure 3. In stage 3 of the extraction, the pressure parameter was increases to 300 bar for cannabinoid extraction. Total extraction time for monoterpenes was 45 minutes, extraction time for cannabinoids was 5.5 hours, and the total efficiency was 92% of the available target compounds were extracted.

Conclusion

In order to develop terpene extraction condition utilising supercritical carbon dioxide, it is essential to understand the relative solubility of terpenes in supercritical carbon dioxide. It was determined that monoterpenes have high solubility at 70 bar and 50°C while sesquiterpenes have low to mild solubility at these conditions. With the addition of a terpene specific collector (CS4), supercritical CO_2 is an effective solvent for the extraction of terpenes from Cannabis. The ability of supercritical carbon dioxide to return to a gaseous state once exposed to ambient conditions allows for simple terpene recovery and results in no detectable residual solvents. Monoterpene fractions were obtained from the terpene specific collector with high purity and no detectable cannabinoids. In order to recover highly volatile monoterpenes, CS4 needed to be chilled to -78°C. Plant waxes and cannabinoids co-elute with sesquiterpenes, which are collected from CS3. Due to the more robust nature of sesquiterpenes, these compounds can undergo post processing methods such as winterization with minimal to no degradation. The ability to separate monoterpenes from sesquiterpenes from cannabinoids allows for post processing of cannabinoids without the danger of terpene degradation.

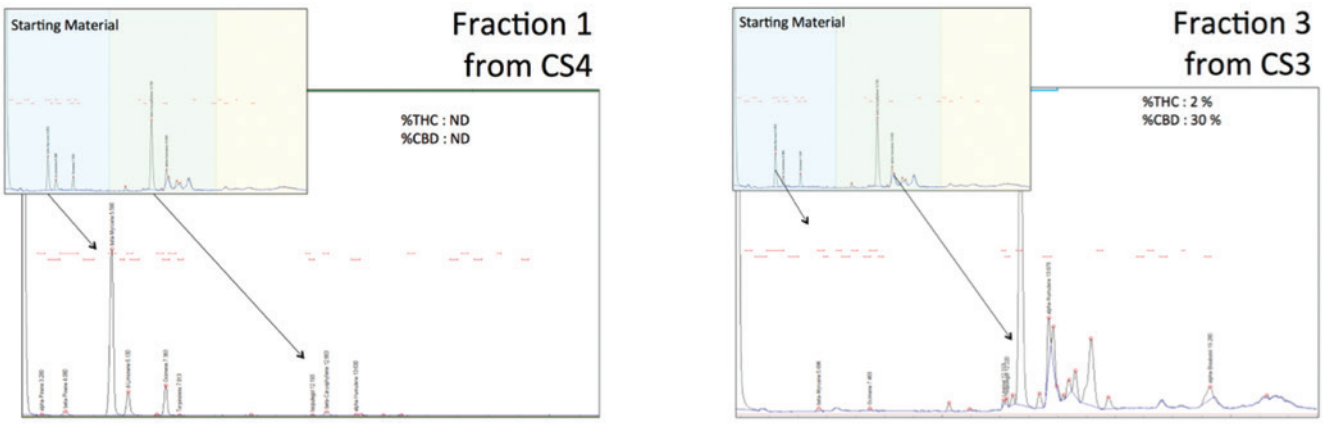


Figure 6: Left chromatogram: Monoterpene fraction obtained at extraction conditions of 70 bar and 50°C. Right chromatogram: Sesquiterpenes obtained at extraction conditions of 150 bar and 55°C.

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