Supercritical Fluid Extraction (SFE) of Monoterpenes from the Leaves of *Melaleuca alternifolia* (Tea Tree)

Victor Wong*, S. Grant Wyllie, Charles P. Cornwell and Deidre Tronson

Centre for Biostructural and Biomolecular Research, Building K12, University of Western Sydney, Hawkesbury Campus, Locked Bag 1797, Penrith South, NSW 1797, Australia.
Tel.: (02) 4570-1640, Fax: (02) 4570-1621.

* Author to whom correspondence should be addressed. E-mail: v.wong@uws.edu.au

Abstract: The technique of supercritical fluid extraction (SFE) was applied to various sample matrices under a range of supercritical carbon dioxide ($scCO_2$) densities and chamber temperatures. The purpose was to develop an effective extraction condition for the removal of eight target monoterpenes from Australian tea tree (*Melaleuca alternifolia* Cheel) leaves. The optimum conditions for extraction were found to be 0.25 g/mL $scCO_2$ density at a chamber temperature of 110^oC. These condition were most effective when applied to **whole fresh** and **rehydrated whole dried leaves**, where it yielded maximum recovery of target analytes with minimum change in oil composition for the extractor system employed. This study demonstrates the importance of the type of sample matrix used in SFE work, and that a different extraction protocol would need to be developed for each matrix.

Keywords: Supercritical Fluid Extraction, SFE, *Melaleuca alternifolia*, Australian Tea Tree

Introduction

SFE is an extraction method where the usual liquid solvent phase has been replaced by a supercritical fluid [1]. A supercritical fluid exhibits physical properties (density, viscosity and diffusivity) that lie between those of a gas and liquid, giving an extraction solvent whose solvating powers are liquid-like while its diffusivity and viscosity are gas-like [2]. By adjusting its density (through pressure and/or temperature modification), a wide range of solvating power can be achieved so that it is possible to extract different components under particular sets of conditions. Supercritical

fluids are therefore suitable extraction solvents for the separation of analytes or classes of compounds from many matrices, including plant tissues [1,2].

Traditional extraction methods such as steam distillation and soxhlet extraction suffer from several limitations: soxhlet extraction usually involves organic solvents that are flammable, toxic and environmentally unfriendly; steam distillation frequently calls for the institution of harsh conditions where some components may be lost through thermal degradation [3], hydrolysis or volatilization. The resultant extract may therefore reflect an incomplete or altered suite of compounds compared with those found in the original material [4].

In view of the shortcomings of traditional extraction methods, SFE is increasingly regarded as an attractive extraction technique because of its rapidity, selectivity, mildness and environmental friendliness. Speed and the ability to automate the extraction process are also important advantages.

By far the most popular supercritical fluid for use in SFE applications is carbon dioxide (CO_2). As a solvent, $scCO_2$ possesses the following benefits: (1) it is a non-toxic and cheap extraction medium involving little or no organic solvents; (2) thermally labile compounds can be safely extracted; (3) extraction conditions can be effectively controlled through temperature and/or pressure modifications; (4) the supercritical state ($scCO_2$) is easy to achieve since CO_2 possesses a critical temperature of $31^{o}C$ and a critical pressure of 7.4 MPa; and (5) it has good nonpolar solvent properties akin to hexane. However, modifiers such as methanol can be introduced to extract polar compounds [1,2,5].

The objective of this study was to evaluate the overall performance of the SFE system, and to develop an optimized set of conditions for the complete $scCO_2$ extraction of eight common monoterpenes from Australian Tea Tree (*Melaleuca alternifolia*) leaves. This included evaluating chamber phenomena, and subsequent trapping and analyte recovery procedures.

Results and Discussion

Composition of Typical Tea Tree Oil

A sample of commercial tea tree oil was analyzed by GC to establish a "benchmark" composition for the target monoterpenes. The eight monoterpenes of interest, which comprise approximately 87% of the tea tree oil, are listed in Table 1, together with their characteristic relative retention times and the percentage contribution of each component to the total 8 monoterpenes.

Table 1. Retention times and composition of the target monoterpenes.

Monoterpenes	Retention Times (min)	% Composition
α-Pinene	6.594	3.10
α-Terpinene	9.309	9.60
para-Cymene	9.666	3.30
1,8-Cineole	9.978	13.4
γ-Terpinene	10.843	20.6
Terpinolene	11.901	4.80
Terpinen-4-ol	15.890	40.6
α-Terpineol	16.484	4.50

Five sample types were used, namely whole dried leaves, crushed dried leaves, whole fresh leaves, crushed fresh leaves, and rehydrated whole dried leaves. Composition of the oil, as reflected in the alteration of ratios between oil components was also assessed. For the purpose of this work, the term "complete extraction" was defined as obtaining at least 95% of target analytes in the first extraction step, bearing in mind that extractions were not expected to be fully efficient.

(A) Model Matrix

(1) Commercial Tea Tree Oil on Filter Paper

Samples of the same commercial tea tree oil were placed on filter paper (a simple matrix) and extracted separately at different $scCO_2$ densities (0.25, 0.40 and 0.60 g/mL) using a chamber temperature (CT) of 40°C. For each density, three successive extractions were performed to assess whether the target components had been extracted completely. Results are shown in Figure 1.
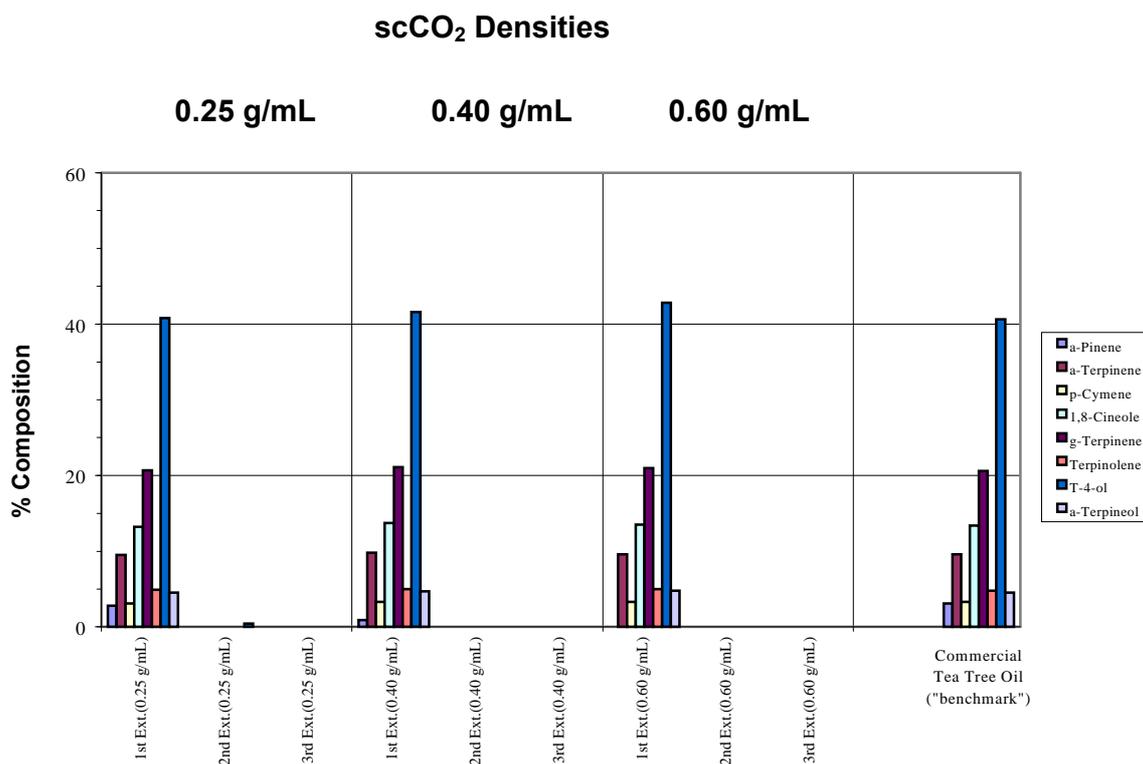
Figure 1. Effects of different conditions on the extraction of commercial tea tree oil from a simple matrix. The chamber temperature was set at 40°C. Note in the chart legend that "g-Terpinene" was used to denote γ-Terpinene, "a-Pinene" represented α-Pinene and "t-4-ol" was used in place of Terpinen-4-ol, and so on. "Ext." has been used to denote extraction. These naming conventions will be adhered to in subsequent charts.

Compared to the "benchmark" (pure commercial tea tree oil), Figure 1 shows that 95% of the target analytes appeared in the extract from the first extraction step. As defined above, this indicated that extraction was complete after one extraction cycle at all the $scCO_2$ densities employed. The ratios between each component were also generally maintained, hence, there was no specific component selectivity observed in the extractions. However, a decreasing concentration of α-pinene was observed as the density of the supercritical fluid was increased such that at 0.60 g/mL $scCO_2$ density, this component was no longer found. This loss may be attributed to the inability of the trap to retain this, the most volatile of the terpenes, during the passage of the large volume of CO_2 gas generated by decompression of high density $scCO_2$.

(B) Real Sample Matrices

(1) Whole Fresh Leaves

Whole fresh leaves were extracted using a chamber temperature of $40^{\circ}C$ and incomplete extractions were observed across all densities (Figure 2a). At a density of 0.25 g/mL, only terpinen-4-ol and some γ-terpinene were extracted. However, selective extraction towards terpinen-4-ol was evident, particularly at higher densities (0.40 and 0.60 g/mL) where no γ-terpinene was extracted, possibly due to the slight change in polarity of the scCO2. It can also be seen that, generally, more terpinen-4-ol was extracted as the supercritical fluid density was increased.
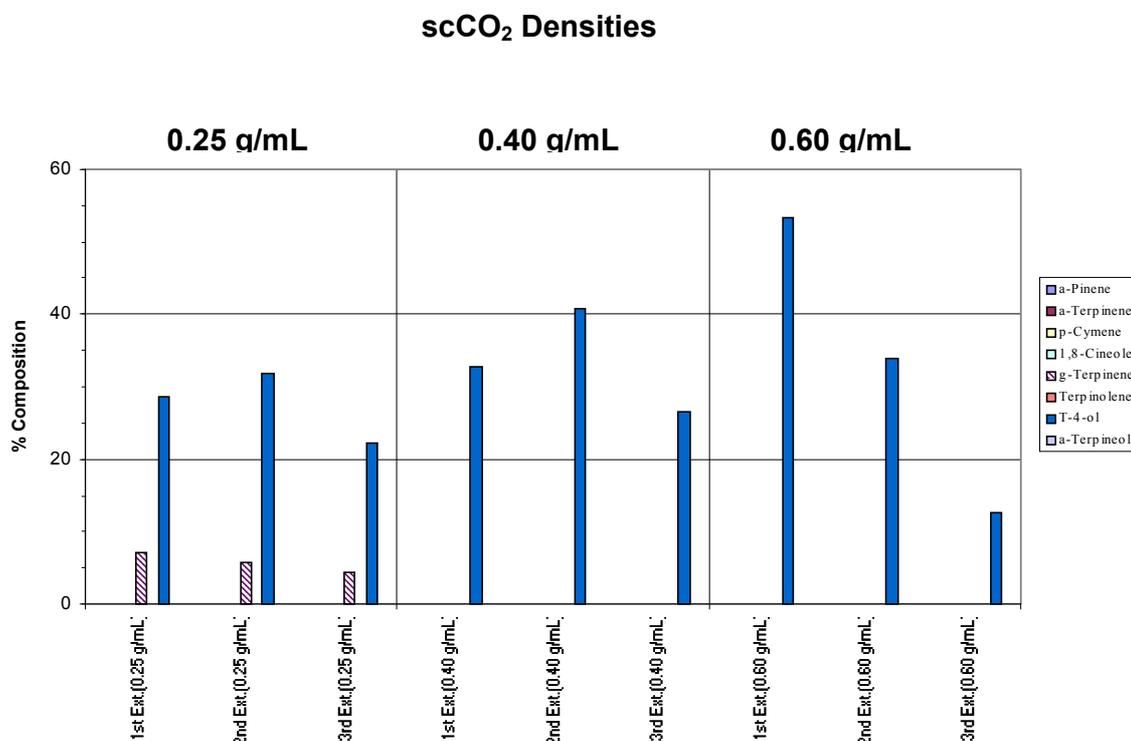
Figure 2a. Extraction of whole fresh leaves at densities of 0.25, 0.40 and 0.60 g/mL and a chamber temperature of $40^{\circ}C$.

Complete extraction, according to our definition, occurred for densities of 0.25 and 0.40 g/mL when a chamber temperature of 80°C was applied (Figure 2b). However, for the sample that was extracted at a density of 0.60 g/mL, incomplete extraction, according to our definition, was observed owing to carry-over related to the higher $scCO_2$ density used. The gradual disappearance of α-pinene in relation to the trap's deficiency with increasing $scCO_2$ density was again evident.
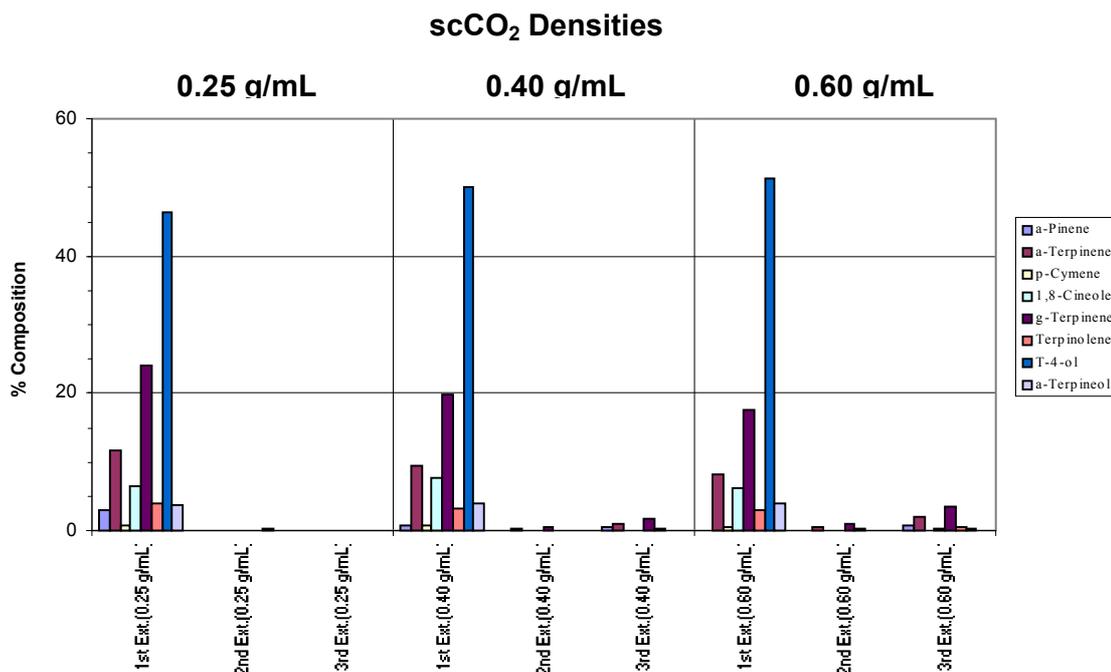
Figure 2b. Recovery of target analytes from whole fresh leaves using a chamber temperature of 80°C.

Compared to the second extraction step, a slight increase in the concentration of components at the third extraction step was noted for the 0.40 and 0.60 g/mL $scCO_2$ densities. This can be attributed to sample carry-over and appears to be a consequence of the final depressurization and line purging sequence automatically initiated by the instrument. This was consistently observed for higher $scCO_2$ density extractions.

(i) Chamber Temperature of 110°C

The results for whole fresh leaves subjected to extraction at a chamber temperature of 110°C are given in Table 2 and Figure 2c.

Table 2. Data for whole fresh leaves extracted at a chamber temperature of 110°C.

Whole Fresh Leaves Chamber Temperature = 110°C								
	% Composition							
scCO$_2$ Densities	α-Pinene	α-Terpinene	p-Cymene	1,8-Cineole	γ-Terpinene	Terpinolene	T-4-ol	α-Terpineol
1st Ext.(0.25 g/mL)	2.2	9.4	1.5	3.4	21.1	3.3	54.5	3.9
2nd Ext.(0.25 g/mL)	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
3rd Ext.(0.25 g/mL)	0.0	0.1	0.0	0.0	0.3	0.0	0.1	0.0
1st Ext.(0.40 g/mL)	0.4	8.6	0.6	3.0	18.7	3.1	52.6	3.7
2nd Ext.(0.40 g/mL)	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
3rd Ext.(0.40 g/mL)	0.9	2.3	0.2	0.2	4.4	0.6	0.4	0.0
1st Ext.(0.60 g/mL)	0.1	6.8	1.4	4.2	17.2	2.7	48.8	3.5
2nd Ext.(0.60 g/mL)	0.0	0.4	0.0	0.0	1.0	0.2	0.2	0.0
3rd Ext.(0.60 g/mL)	1.3	3.2	0.3	0.4	6.4	0.9	1.1	0.0

scCO$_2$ Densities
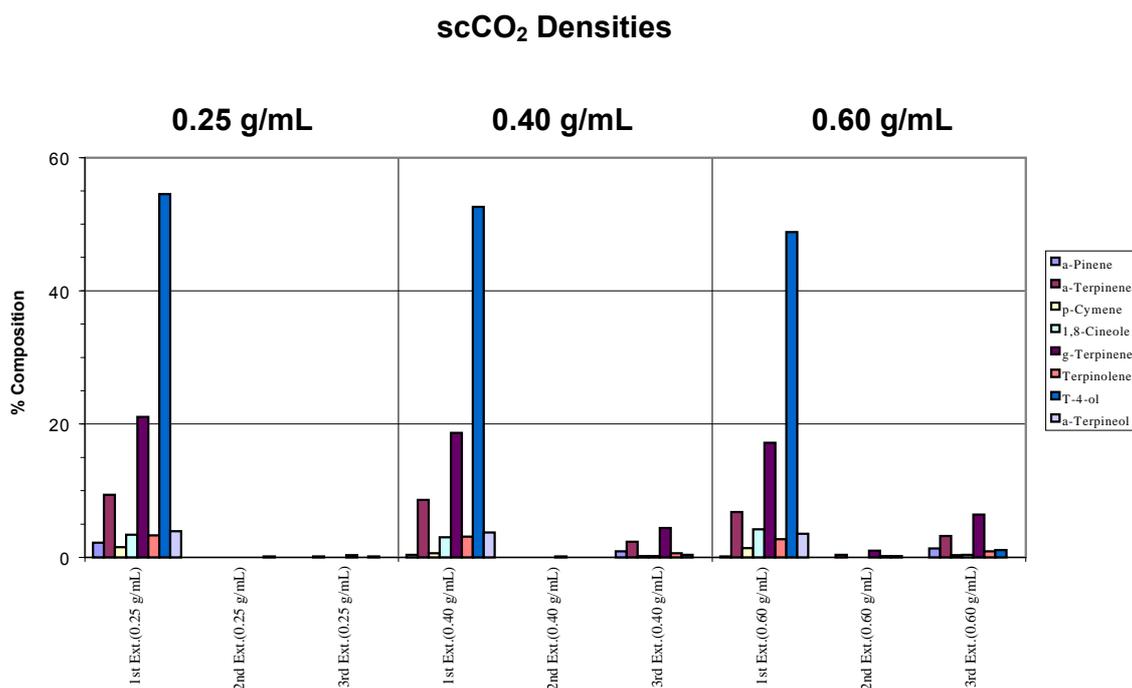
Figure 2c. Recovery of target analytes from whole fresh leaves using a chamber temperature of 110°C.

If the sum total of each analyte for the three extractions at 0.4 and 0.6 g/mL were compared to that of the 0.25 g/mL extractions, very little difference was noted. Therefore, in terms of efficiency, which includes the ability to trap, all three extraction densities yielded similar results. However, sample carry-over, as observed above, was apparent for the 0.4 and 0.6 g/mL extractions. In addition, the gradual disappearance of α-pinene was again observed. For these reasons, 0.4 and 0.6 g/mL extraction densities

were not considered optimal. To yield better extraction efficiencies under these conditions, a larger capacity trap is required. Without such a trap, a $scCO_2$ density of 0.25 g/mL proved to be the most efficient at this chamber temperature, in accordance with the project's definition, for this particular instrument. This chamber temperature also possesses the added feature of the micro-steam distillation effect and the presumed role of the moisture or steam to act as a modifier in enhancing the extraction process.

The established optimum extraction condition was subsequently applied to a different batch of leaf material to determine if similar results could be achieved. Comparison with the above experiment showed similar results. Therefore, the optimum extraction condition was considered to yield reproducible results when applied to whole fresh leaves.

The fact that the optimum extraction conditions were able to reproducibly achieve complete extraction when applied to whole fresh leaves does not necessarily mean that all the target analytes have been removed from the sample matrix. More analytes may be extracted when harsher conditions were applied. To assess this possibility, a sample was extracted twice using the optimum conditions, and then the $scCO_2$ density was immediately increased from 0.25 g/mL to 0.60 g/mL for two further extractions.

It was found that (data not shown) no significant additional amounts of terpenes were extracted and that the optimum conditions resulted in almost complete extraction and recovery of the target analytes from whole fresh leaves.

(2) Whole Dried Leaves

Whole dried leaves remain the preferred working matrix from an analyst's point of view, particularly when a large population of samples needs to be analyzed. Therefore, the most convenient option is to collect the samples, air dry and then store them in an open and/or relatively dry location pending extraction. Whole dried leaves were extracted at scCO2 densities of 0.25, 0.40 and 0.60 g/mL and at chamber temperatures of 40°C, 80°C and 110°C. Incomplete extractions were observed in all cases, with significant concentrations of components still detected even after the third extraction step for all densities and chamber temperatures employed. For the experiment involving a chamber temperature of 110°C, a graphical representation of the results is provided in Figure 3.

scCO$_2$ Densities
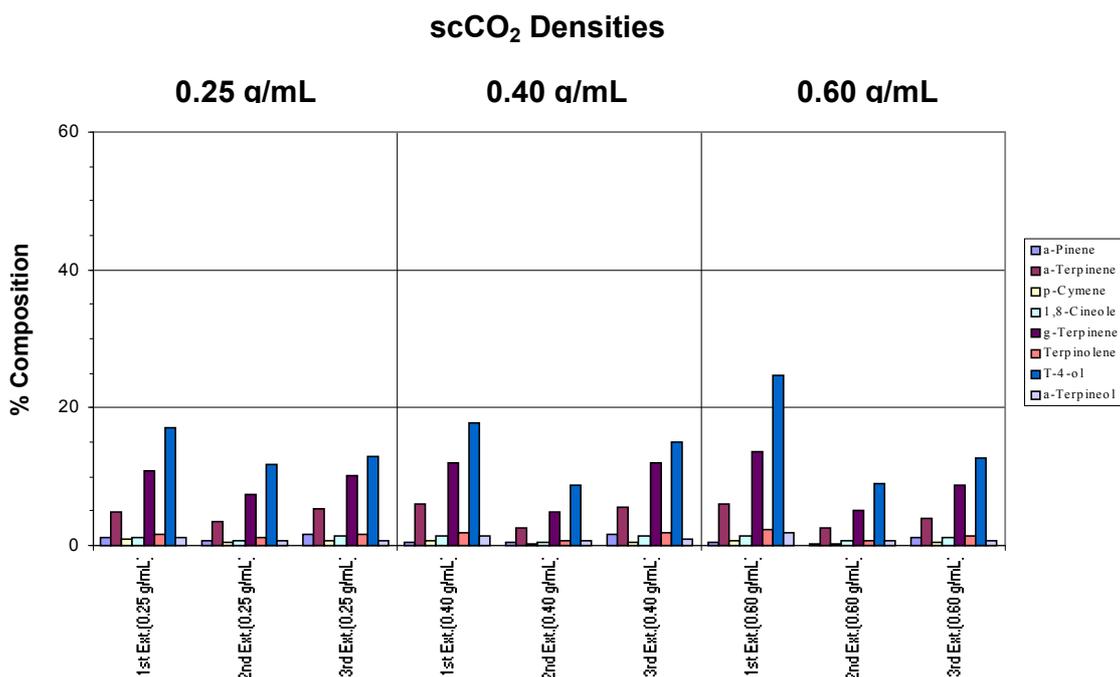
Figure 3. Recovery of target analytes from whole dried leaves using a chamber temperature of 110°C.

(3) Rehydrated Whole Dried Leaves

Rehydrated whole dried leaf samples were used to determine the role of water in the extraction process. Complete extraction was achieved with the rehydrated sample when the optimum extraction condition was applied (Figure 4). The oil composition was also very similar to that obtained from the original whole fresh leaves extraction (Figure 2c). The few components that appeared in the second and third extraction steps for the rehydrated sample were relatively low in concentration, again, reflecting the sample carry-over characteristic of the instrument.
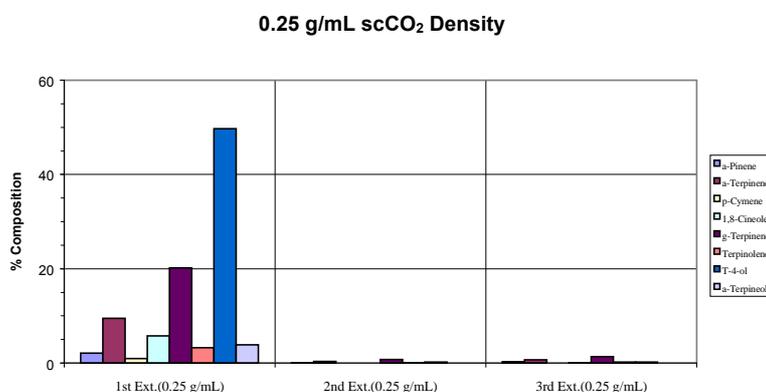
Figure 4. Recovery of target analytes from rehydrated whole dried leaves employing the optimum extraction conditions.

(4) Crushed Dried Leaves

Crushed samples were also investigated. As observed in whole dried leaves, incomplete extractions occurred under all chamber temperatures and densities combinations (data not shown).

(5) Crushed Fresh Leaves

The extraction of crushed fresh leaves was investigated to see if the sample preparation could influence extraction conditions. The results showed that the conditions established for whole fresh leaves also gave the best results with crushed fresh leaves, i.e. crushing did not influence extraction efficiency in this case (Figure 5).
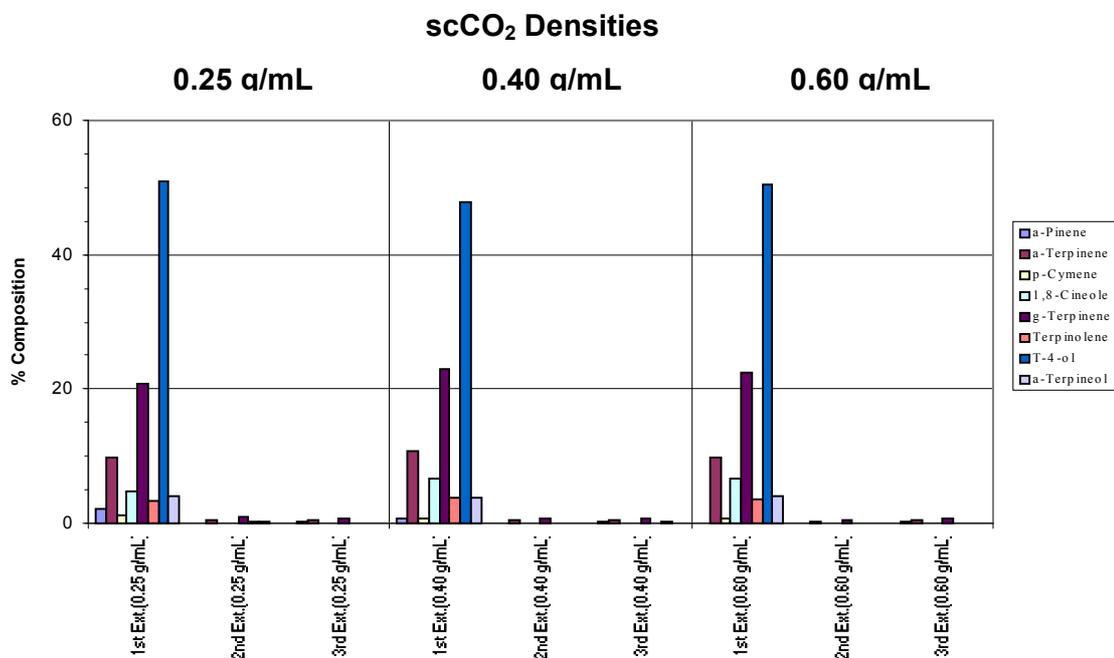
Figure 5. Recovery of target analytes from crushed fresh leaves using a chamber temperature of 110°C.

Conclusions

The complete extraction of tea tree oil from a model matrix under very mild conditions was not duplicated with real leaf sample matrices. However, under more vigorous conditions complete extraction of the eight target monoterpene analytes from fresh and rehydrated dried leaf samples was achieved. These results emphasize the importance of the sample matrix to the extraction process and the possible influence of the presence of a modifier such as water on supercritical CO_2 extractions.

Experimental

Chemicals

Carbon dioxide, SFE and normal grades, were purchased from BOC Gases (Sydney, Australia). *n*-hexane and absolute ethanol (analytical-reagent grade) were obtained from Biolab Scientific (Sydney, Australia).

Samples

Leaf samples (*M. alternifolia*) were randomly obtained from a small plantation at the Horticulture Precinct, Faculty of Science, Technology and Agriculture, University of Western Sydney, Hawkesbury, Sydney, Australia. For experiments involving dried leaf samples, the leaves were air dried at room temperature over a period of at least two weeks before any extraction work was carried out. For fresh leaves, samples were extracted within an hour of sampling. A commercial tea tree oil sample was provided courtesy of ATTORI (Australian Tea Tree Oil Research Institute, Lismore, Australia).

Sample Crushing

Leaf material was transferred into a mortar and a sufficient quantity of liquid nitrogen added. Grinding with a pestle was undertaken until a coarse appearance resulted.

Sample Rehydration

A sufficient quantity of the leaf material was immersed in water overnight. On the following day, the sample was blotted dry prior to extraction.

Supercritical Fluid Extractor and Extraction Conditions

A Hewlett Packard (HP) 7680T SFE instrument, with accompanying software, was used throughout this study as an integrated system of extraction, subsequent trapping and analyte recovery. A known amount of the leaf material (approximately 100 mg) or a drop of the oil on filter paper was loaded into the sample chamber (thimble) between glass-fiber impregnated filter papers. Extraction procedures involved the manipulation of only two parameters for each sample type, (1) the chamber temperature and (2) the $scCO_2$ density. For every chamber temperature/$scCO_2$ density combination employed, three successive extractions were performed on the same sample.

A complete list of extraction parameters are outlined in Table 1. The extraction time (dynamic extraction) parameter in Table 1 was listed in terms of the number of thimble volume sweeps. The actual time required for this extraction mode varies with the $scCO_2$ density used, hence the volume sweep number is more informative for this work. The trap temperature was set at 5°C to minimize the loss of volatile components.

Table 3. SFE parameters that were maintained in all extractions.

Extraction Conditions	Values
CO_2 flow rate (mL/min)	1
Equilibrium time (min)—Static Extraction	10
Extraction time —Dynamic Extraction in number of times thimble volume sweeps	10 times
Nozzle extraction temperature (^{o}C)	45
Trap extraction temperature (^{o}C)	5
Trap packing	ODS (#)
Rinse Conditions	
Nozzle rinse temperature (^{o}C)	45
Trap extraction temperature (^{o}C)	5
Rinse solvent	Hexane
Rinse solvent flow rate (mL/min)	2
Rinse solvent volume (mL)	1.5
Void volume compensation (mL)	1.04

Octadecylsilane

Chromatographic Conditions

All chromatographic analyses were performed on a Model HP6890 gas chromatograph, fitted with a HP7693 auto-injector, using a SGE 50QC2/BPX5 cross-linked polydimethylsiloxane column (50 m length x 220 μm ID x 0.25 μm film thickness). Oils and extracts were analyzed under the following conditions:- injection volume: 2 μL, inlet temperature and pressure: 280°C and 138 kPa respectively, carrier gas: hydrogen at 40 mL/min, split ratio 1:30, detector temperature: 280°C, temperature program: initial temperature, 80°C for 5 minutes, increased to 180°C at 4°C/min, then to 300°C at 25°C/min and maintained at the final temperature for 5 minutes. Data obtained were stored and processed using HPChemStation software.

Acknowledgments

References and Notes

1. Bevan, C. D.; Marshall, P. S. The Use of Supercritical Fluids in the Isolation of Natural Products. *Natural Product Reports* **1994**, *32*, 451-466.

2. Phelps, C. L.; Smart, N. G.; Wai, C. M. Past, Present and Possible Future Applications of Supercritical Fluid Extraction Technology. *Journal of Chemical Education* **1996,** *73*, 1163-1168.
3. Reverchon, E.; Senatore, F. Supercritical Carbon Dioxide Extraction of Chamomile Essential Oil and Its Analysis by Gas Chromatography-Mass Spectrometry. *Journal of Agriculture and Food Chemistry* **1994**, *42*, 154-158.
4. Reverchon, E.; Della Porta, D.; Senatore, F. Supercritical CO_2 Extraction and Fractionation of Lavender Essential Oil and Waxes. *Journal of Agriculture and Food Chemistry* **1995**, *43*, 1654-1685.
5. Vannoort, R. W.; Chervet, J. P.; Lingeman, H.; DeJong, G. J.; Brinkman, U. A. Th. Coupling of Supercritical Fluid Extraction with Chromatographic Techniques. *Journal of Chromatography* **1990**, *505*, 45-77.

Samples Availability: Available from the authors.