

**Characterization of essential oils by comprehensively coupled  
supercritical fluid and gas chromatography  
(SFCxGC)**

BY

**PETER RAMASHADI MAKGWANE**

Supervisor

Prof. E. R. Rohwer

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**SUMMARY**

Essential oils are amongst the most complex samples an analyst can face in terms of the number of compounds involved. In many cases, minor components are of interest as they can impart a distinctive fragrance character to the oil. Because of the closely related structures and molecular weights among terpenes, positive identification of individual compounds is very difficult with a single chromatographic technique. Further, most of the analytical information is lost when a single technique is used because of the limited peak capacity and the resulting peak overlap. For many years, gas chromatography coupled to mass spectrometry (GC-MS) has been the benchmark

technique for qualitative and quantitative analysis of essential oils. Retention indices and mass spectra have to be used in combination for confirmation of the identity of components in an essential oil. Other multidimensional or hyphenated techniques also offer advantages that aid in the identification of essential oil components. This thesis demonstrates the application of a comprehensively coupled supercritical fluid and fast temperature programmed gas chromatograph (SFCxGC) to the analysis of essential oils. An SFCxGC instrument was used to analyse the essential oils of *Cymbopogon* (lemongrass), *Artemisia afra* (wilde als), *Tagetes minuta* (kakiebos) and *Pelargonium* (geranium) species. The unique application of a porous layer open-tubular (PLOT) column, used in conjunction with supercritical carbon dioxide is demonstrated to effect group separation of polar, oxygenated compounds. This separation and elution of very polar compounds from a silica gel column is believed to occur due to the reduced phase ratio ( $\beta$ ) of the system obtained by increasing the volume available to the mobile phase compared to that of a packed column. This separation obtained in the SFC is used to separate essential oils into different chemical classes such as non-polars, ethers, alcohols. Separated chemical classes are re-injected on-line by use of a modulator into a fast, second dimension, temperature programmed GC to effect separation of individual compounds based on their volatility. The entire sample is analysed by both the SFC and GC in such a way that the resolution obtained in the first dimension is conserved by the GC analyses. By using a range of standards, some of the peaks in these oils could be assigned. The identification of compounds was greatly aided by the combination of the two separation dimensions. The comprehensive two-dimensional technique arranges component peaks in a plane from which chemical class and volatility information of each component is readily obtained. The elution pattern within the two-dimensional chromatograms may also be used for direct comparison of oils without identification of the components in the essential oils.

**Die karakterisering van essensiele oliës met omvattend-gekoppelde  
superkritiese-fluïed-en gas chromatografie  
(SFCxGC)**

Deur

**PETER RAMASHADI MAKGWANE**

Promotor

Prof. Dr E R Rohwer

Ingehandig in deelse bereik van die behoeftes vir die graad

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**Samevatting**

Vlugtige plantolies is van die mees komplekse monsters waarmee 'n analis te doen kan kry wat betref die aantal verbindings betrokke. In baie gevalle is lae-voorkoms komponente van belang aangesien hulle 'n karakteristieke geur kan verleen aan die olie. As gevolg van die nou-verwante strukture en molekulêre massas van terpene, is positiewe identifikasie van komponente baie moeilik met 'n enkele chromatografiese tegniek. Verder is meeste van die analitiese inligting onbekombaar met 'n enkele tegniek, as gevolg van beperkte piek-kapasiteit en die gevolglike oorvleueling van

komponente. Gaschromatografie en massaspektrometrie was vir baie jare die staatmakertegnieke vir die kwalitatiewe en kwantitatiewe ontleding van vlugtige olies. Gewoonlik moet retensie-indekse en massaspektra saam gebruik word (GC-MS) vir bevestiging van die samestelling van 'n vlugtige olie. 'n Superkritiese-vloeistofchromatograaf, omvattend gekombineer met 'n vinnige temperatuur-geprogrammeerde gaschromatograaf (SFCxGC) word gebruik om vlugtige plantolies te ontleed uit die volgende plante: *Cymbopogon* (sitroengras), *Artemisia afra* (wilde-als), *Tagetes minuta* (kakiebos) en *Pelargonium* (malva). 'n Poreuse-laag, oop-buis-kolom (PLOT-kolom) met 'n klein faseverhouding ( $\beta$ ) word in die SFC-dimensie gebruik om die monster te skei in verskillende chemiese klasse. Daarna word die geskeide klasse direk deur middel van 'n modulator ingelaat in 'n vinnige, tweede-dimensie temperatuur-geprogrammeerde gaschromatograaf om individuele komponente te skei op grond van hul vlugtigheid. Deur die gebruik van 'n reeks standaarde kan sommige van die komponente geïdentifiseer word. Vanuit ons kennis van die hoofkomponente in hierdie olies en hul retensie-gedrag, kan die meeste pieke uitgeken word met die twee-dimensionele SFCxGC chromatogramme. Dit blyk dat daar 'n elueringspatroon is in die twee-dimensionele chromatogramme wat gebruik kan word om olies te vergelyk en om onbekende stowwe in vlugtige plantolies uit te ken.

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**ABBREVIATIONS**

FID	:	Flame Ionization Detector
GC	:	Gas Chromatography
GCxGC	:	Comprehensive two-dimensional gas chromatography
GC-MS	:	Gas Chromatography-Mass Spectrometry
GC-TOF-MS	:	Gas Chromatography-Time-of Flight- Mass Spectrometry
HETP	:	Height Equivalent of the Theoretical Plate
LCxLC	:	Comprehensive two-dimensional liquid chromatography
MS	:	Mass spectrometry
PLOT	:	Porous Layer Open-Tubular
SFC	:	Supercritical fluid chromatography
SFCxGC	:	Comprehensive two-dimensional supercritical fluid and gas chromatography
SPME	:	Solid-Phase Microextraction
SMO	:	Statistical Model of component Overlap
LRI	:	Linear Retention Index

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# CHAPTER 1

## GENERAL INTRODUCTION

### 1.1 Background

Essential oils are amongst the most complex mixtures an analyst can face in terms of the number of compounds involved. An estimated 1,200 compounds, including terpenes and their corresponding aldehydes, ketones, alcohols, phenylpropanoids, hydrocarbons, esters, oxides and sulfur compounds, have been identified in essential oils<sup>1</sup>. Essential oils are defined as volatile plant products whose constituents are a complex mixture of terpenic hydrocarbons and oxygenated derivatives such as aldehydes, ketones, esters and alcohols. They contain volatile compounds of plant origin with unique properties that have been prized worldwide for thousands of years.

Because of the enormous amount of plant material required to produce natural essential oils, products in the market are often adulterated with lower quality, commercial grade oils or synthetic compounds. These reduce the costs in order to increase the profit margin, a fact not usually revealed on the label. Issues concerning essential oil adulteration and the dilution of the original oils with those of lesser value have also been investigated<sup>2,3</sup>.



Oils from thousands of plant species have been extracted and are commercially available<sup>4,5</sup>. Essential oils are extracted from plant materials by a number of procedures including steam distillation, vacuum distillation, solvent extraction, cold-pressing and hot-pressing<sup>6</sup>. The composition of these oils can vary significantly with place of origin, harvest season, and climate. A common feature is that essential oils possess the essence of some plant, the identifiable aroma, flavour or other characteristic that has some practical use. They are used in cosmetics (perfumes), food flavours, deodorants, pharmaceuticals and embalming antiseptics.

Because of the incredible complexity and hundreds of different chemical constituents contained in one single oil, it becomes clear that analysis of essential oils is difficult. To add to the complexity of the volatile oils, the time of harvest, climate, the soil and the mode of essential oil extraction influences the oil composition and consequently the amount of biologically active substances<sup>7</sup>. The quality and price of some oils are based on the percentage content of some components contained in the oil, so separation and measurement of these components are very important. This is usually done using chromatography and spectroscopy. Gas Chromatography (GC) and GC coupled to mass spectrometric detection (GC-MS) has been used to ascertain quality and purity of most essential oils<sup>8,9</sup>.

Recently, the greatest efforts have been directed towards improving methods in order to obtain better separation, especially for complex samples, at lower cost and faster speed. The identification of components based on only one parameter, typically retention time, has become inadequate for complex mixtures. A different strategy for achieving unequivocal identification of compounds, is to increase the number of parameters that can be used simultaneously in detection<sup>10</sup>.

The use of the linear retention indices (LRI) and mass spectra data for essential oil compositional analysis was developed into an index<sup>11</sup> that combines the two criteria for final identification of the compounds. The use of either mass spectral results or LRI alone may lead to erroneous results.

A difference in mass spectra may be observed if the spectra were obtained using an ion trap MS<sup>12</sup>. Often different spectra are reported in the MS library for one component peak. The spectral similarity of a great number of essential oil components often precludes positive identification of individual components. Mass spectra for many sesquiterpenes are identical or nearly the same<sup>13</sup>. It has been found that more than 230 naturally occurring sesquiterpenes have a molecular mass of 204<sup>11</sup>. Since many of these sesquiterpenes may be present in the same essential oil it is very difficult if not impossible to positively separate and identify all components in an oil with a one-dimensional technique. Compilations such as that of Adams<sup>5</sup> suggest standard conditions that other researchers may use to identify the chromatographic peaks in the chromatogram.

Multidimensional techniques offer a solution to this problem by providing more resolving power, resulting in enhanced peak capacity, selectivity and a larger number of parameters for the characterisation and identification of components in complex mixtures<sup>10</sup>. In many cases the dimensions are two or more chromatographic steps, or a chromatographic separation with spectroscopic detection.

Multidimensional liquid chromatography-gas chromatography (LC-GC), in which an HPLC is coupled on-line with GC, gives lots of information concerning a sample in a single run. Therefore, multidimensional HPLC-GC is useful for the analysis of complex samples, such as natural products (essential oils)<sup>14,15</sup>. HPLC offers chemical class separation of compounds. One of the problems in the HPLC-GC system is the large volumes of the HPLC mobile phase that need to be removed when introduced into the GC injector<sup>16</sup>. Pre-treatment or clean-up of samples is important, before injection into the analytical system. This can be labour-intensive and time consuming. Therefore, there is a high demand for on-line systems that can do pre-separation of complex mixtures into groups, thus reducing the complexity of the sample matrix before detailed on-line analysis of individual compounds in each group to yield valuable information in a short time.

Terpenic hydrocarbons are unstable to heat and light and they degrade to produce compounds with undesirable off-flavours when exposed to light or heat for long a time. Furthermore, terpene hydrocarbons do not contribute much to the flavour or fragrance of the oil, even at higher concentrations<sup>13</sup>. The oxygenated compounds such as aldehydes, esters and alcohols determine the characteristic flavour and odour of essential oils so valuable for their applications in a number of industries. These oxygenated groups are difficult to elute on silica- gel due to large retention factors when using the fairly non-polar CO<sub>2</sub> as mobile phase only. However, with SFC using a porous layer open tubular (PLOT) column, oxygenated compounds are eluted<sup>17</sup>.

In South Africa, *Cymbopogon citratus* & *flexuosus* (lemongrass), *Tagetes minuta* (kakiebos), *Artemisia afra* (wilde als) and *Pelargonium* (geranium) plants, among others, are grown for commercial production of essential oils. Analysis of these oils is important to the farmers for a number of reasons, for example, quality control purposes. Bioprospecting for new oils in indigenous plants is also of interest. Therefore, there is a need for improved analytical techniques that would provide valuable information at lower cost and in a reduced time.

## 1.2 Approach

The main purpose of the study is to investigate the potential of SFCxGC in fingerprinting essential oils. A successful fingerprinting technique should produce sufficient, reproducible information to discriminate between oils of closely related species. It should also discriminate between oils from the same species of different geographical origin. This technique provides a new solution for the analysis of complex essential oils by increasing the selectivity of the separation method and reducing peak overlap.

In SFCxGC, mixtures of compounds are subjected to two independent separation dimensions. In the first dimension compounds are separated into different chemical classes using SFC with supercritical carbon dioxide as mobile phase. The separated groups are further separated into individual compounds in the fast temperature programmed GC based on their different boiling points. The combination of the two parameters (polarity and volatility) aids assignment of compound identity.

Supercritical fluid chromatography (SFC) is a separation technique that bridges GC and LC. SFC uses the chemical class separation capability of normal phase liquid chromatography. The availability of gas chromatographic flame ionization detector (FID) simplifies the use of SFC as compared to LC especially for compounds without a chromophor. The use of SFC for group separation of essential oils has been reported<sup>18,19</sup>.

A comprehensive two-dimensional SFCxGC system provides a substantial increase in peak capacity by serially coupling two separation mechanisms for the analysis of natural products (essential oils). A flow modulator (using the stop-flow principle) interfaces the SFC to the fast temperature- programmed GC, facilitating the on-line transfer of eluents from the SFC into the fast GC. The modulator cuts the entire sample stream from the SFC into consecutive slices and re-injects them into a fast second dimension for further analysis. The resulting two-dimensional chromatograms provide information relating to both the chemical class and volatility of the components in a sample and greatly aids the identification of unknown compounds in complex mixtures.

### 1.3 Presentation and arrangement

Each chapter deals with a separate aspect of the research and has its own references (found at the end of each chapter).

**Chapter 2** explains the production of essential oils and methods of isolation, followed by a brief discussion of essential oil plant material studied in this work.

**Chapter 3** is devoted to methods for essential oils analysis. Starting from one-dimensional analysis to coupled techniques, including comprehensively coupled techniques. The fundamental concepts of multidimensional chromatography and fast GC are also introduced. This will allow better understanding of the theory and operational procedures of comprehensive two-dimensional supercritical fluid and fast temperature programmed gas chromatograph (SFCxGC).

**Chapter 4** looks at theoretical consideration and physico-chemical properties of supercritical fluids as mobile phases in chromatography.

**Chapter 5** covers the instrumental aspects of SFC and optimisation of the system parameters (mobile phase flow rates, temperature, and pressure) for group-type separation of essential oils. It also suggests how the unique elution and separation of the PLOT column can be explained.

**Chapter 6** describes in detail the instrumentation of comprehensive two-dimensional SFCxGC. Problems experienced with the system are discussed, as well as the potential of the SFCxGC for the analysis of complex mixture such as essential oils.

**Chapter 7** provides the conclusion to the project.

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# **CHAPTER 2**

## **PRODUCTION AND ISOLATION OF ESSENTIAL OILS**

### 2.1 Introduction

Essential oils have been known to mankind for centuries. For all these years, extracting the odours from plants has been an important occupation. This has developed into a large modern industry. Essential oils are important raw materials for many industries where they play a number of roles. By definition essential oils contain highly volatile substances that are isolated by a physical method or process from plants of a single botanical species<sup>1</sup>. The oils normally bear the name of the plant species from which they are derived. Essential oils are so termed as they are believed to represent the very essence of odour and flavour.

Essential oils are composed of different chemical groups of terpenic hydrocarbons and their oxidized derivatives such as aldehydes, esters, ketones and alcohols. Terpenes represent a large group of natural compounds that do not contribute much to flavour, fragrance or odour of the oil<sup>2</sup>. Very often the hydrocarbon terpenes represent a large percentage of the components of essential oils of plants and can be found in a remarkable variety of closely related structures. As a common feature, essential oils carry the essence



of a plant, the identifiable aroma, flavour or other characteristics that may have some practical use. They are used for different purposes such as<sup>3</sup>:

1. In pharmaceutical products the oils are used for medicinal and cosmetic purposes.
2. In the perfume industry, they are used as the constituents of expensive fragrances.
3. In the food industry, they are used as food preservatives and flavour enhancers.

Often rare and expensive pure oils in the market are diluted with lower quality commercial-grade oils or synthetic chemicals to increase the volume and therefore the profit margin, a fact not usually revealed on the label<sup>4</sup>. This problem can be addressed by developing reliable analytical methods for the detection of adulterated oils in order to discourage or penalise this action.

## 2.2 Isolation techniques of essential oils

The recovery of essential oil (the value added product) from the raw botanical starting material is very important since the quality of the oil is greatly influenced during this step. There are a variety of methods for obtaining volatile oils from plants. Steam distillation, aqueous infusion, solvent-extraction, cold or hot expression and supercritical fluid extraction (SFE) with carbon dioxide are methods often used. The chemical composition of the oil, both quantitative and qualitative, differs according to the technique used to remove the oil from the plant<sup>5</sup>. A comprehensive review of various techniques employed to recover the essential oil from the materials in which they occur was prepared by Weurman<sup>6</sup>. These techniques are briefly explained in this chapter.

### 2.2.1 Steam distillation

The vast majority of true essential oils are produced by the steam distillation method. However, there are various distillation methods that are used. In all of the distillation processes, water is heated to produce steam that carries the most volatile aromatic materials along with it. These aromatic components are then cooled in a condenser and collected in the resulting distillate<sup>4</sup>. During distillation the boiling water penetrates the plant tissue and dissolves a part of the essential oil present in the oil containing structures. This aqueous solution diffuses through the cell membranes and, upon its arrival at the surface, the oil is immediately vaporized<sup>5</sup>. This process continues until all the enclosed volatiles are removed from the cells.

Koedem and co-workers<sup>7,8</sup> investigated the influence of the length of the distillation period on the composition of the essential oils in the seed of the *Unbelli Fenous* species. It was found that the time required to remove the oils from the plant material will have an influence on the quality of the final oil. They established that the composition of the oil changes indirectly during the distillation process. The higher boiling oxygenated compounds are the first to appear in the distillate, whereas the amount of the lower boiling hydrocarbons gradually increases as the distillation process proceeds.

A modified dimension in the recovery of essential oils with the distillation method was the development of a simultaneous distillation/solvent-extraction apparatus by Likens and Nickerson<sup>9</sup>. The device has the major advantage of a many thousand-fold concentration of volatiles from aqueous media in a single step. The greatest interest in this apparatus is evidenced by its wide application in numerous laboratories. Several modified version of this method have been constructed<sup>10</sup>. As the oxygenated oil constituents are much more soluble in boiling water than their hydrocarbon analogues, the latter remain associated with the plant material to a greater extent. In conclusion, it was established that the components of the essential oils are liberated according to their degree of water solubility rather than following their order of boiling point<sup>5</sup>.

Also the presence of trace metal was found to influence the quality of the oil<sup>11,12</sup>. Essential oils come from various parts of plants - the seeds, bark, leaves, stems, roots, flowers, and fruit. The oils can be distilled from the plant material or extracted. The majority of essential oils are distilled using this method. The key to producing a therapeutic-grade essential oil is to preserve as many of the delicate aromatic compounds within the essential oil as possible - elements that are very fragile and destroyed by high temperature and high-pressure. Contact with chemically reactive metals (i.e., copper or aluminum) is another danger to the fragile aromatic compounds in oils<sup>13</sup>. To ensure a high grade of essential oil, it is imperative to use stainless steel cooking equipment at low pressure and low temperature for long periods of time.

### **2.2.2 Solvent-Extraction**

In the solvent-extraction method of essential oils recovery, an extracting unit is loaded with perforated trays of essential oil plant material and repeatedly washed with the solvent. Hexane is often used. All the extractable material from the plant is dissolved in the solvent<sup>5</sup>. This includes highly volatile aroma molecules as well as non-aroma waxes and pigments. The extract is distilled to recover the solvent for future use. The waxy mass that remains is known as the *concrete*. The concentrated concretes are further processed to remove the waxy materials which dilute the pure essential oil. To prepare the *absolute* from the *concrete*, the waxy concrete is warmed and stirred with alcohol (ethanol). During the heating and stirring process the concrete breaks up into minute globules. Since the aroma molecules are more soluble in alcohol than the waxes, an efficient separation of the two results.

Solvent-extraction of essential oils from plant materials using low boiling solvents has an advantage over distillation because the temperature remains relatively low. Usually temperatures below 50 °C are used during most processes<sup>5</sup>. The oils that result from solvent extraction often have a more “natural” composition compared to distilled oils, which may have undergone thermally induced alterations. The formation of artefacts may

negatively influence the quality of extracted volatiles. Schnelle and Horster<sup>14</sup> detected several artefacts among volatiles extracted from the essential oil of a mint species when a mixture of petroleum ether and acetone was used for extraction. It was discovered that these substances were generated by the reaction of the acetone used in the extraction procedure with non-terpenoids present in the plant material.

### **2.2.3 Supercritical fluid extraction**

Supercritical fluid extraction (SFE) is a solvent extraction process that uses a supercritical fluid as the extraction solvent. The low viscosity of supercritical fluids (SFs) combined with high diffusion rates are ideal for the extraction of diffusion-controlled matrices such as plant tissues<sup>15</sup>. Supercritical extraction is faster than liquid extraction and the supercritical fluid solvents are more easily removed. Recovery can be effected by reducing the pressure to release the solvent from the extracted analytes.

Mostly, CO<sub>2</sub> is used as the extraction solvent. CO<sub>2</sub> has the desirable property that it behaves like a solvent and can be manipulated to obtain differential or sequential fractions. Because of its high vapour pressure at room temperature and atmospheric pressure, all traces of gas can easily be removed from the volatile oil. The low critical temperature of CO<sub>2</sub> is particularly useful when extracting oils that contain heat labile compounds<sup>16</sup>. Supercritical fluid CO<sub>2</sub> is inert and does not introduce artifacts. These desirable properties ensure that essential oils are produced that have organoleptic properties closely resembling those of the plant from which they were extracted<sup>17</sup>.

The solvating power of supercritical CO<sub>2</sub> may be improved by the addition of a more polar modifier such as methanol, but it is generally not necessary for the extraction of essential or volatile oils. When temperatures below the critical temperature of CO<sub>2</sub> are used, liquid CO<sub>2</sub> may be used as a very inert, safe liquid solvent which will extract the aroma molecules in a process similar to solvent-extraction<sup>18</sup>.

## 2.3 Essential oil plants

Many plants are grown by South African farmers for the production of essential oils. These include exotic species such as *Cymbopogon* (lemongrass) and *Tagetes minuta* (kakiebos) or indigenous species such as *Artemisia afra* (wilde als) and *Pelargonium* (geranium). The analysis of these oils is important for quality control purposes and also to find specific compounds that have biological activity, for their isolation and pharmaceutical application. South Africa also has a treasure of uncharted botanical riches. New methods need to be developed to expedite the bio-prospecting of our botanical diversity for novel plants with commercial value as future essential oils or other value added products.

In this thesis the essential oils of *Cymbopogon* (lemongrass) *Artemisia afra* (wilde als), *Tagetes minuta* (kakiebos) and *Pelargonium* (geranium) were studied using a novel analytical technique called comprehensive multidimensional supercritical fluid and gas chromatography (SFCxGC).

### 2.3.1 *Artemisia afra* (Wilde als)

*Artemisia afra* is one of the oldest known indigenous medicinal plants in Southern Africa. Its common names in South Africa are: wild wormwood, African wormwood (Eng.); wilde-als (Afr.); umhlonyane (Xhosa); mhlonyane (Zulu); lengana (Tswana); zengana (Southern Sotho). *Artemisia afra* grows in thick, bushy, slightly untidy clumps, usually with tall stems up to 2 m high, but sometimes as low as 0.6 m. The stems are thick and woody at the base, becoming thinner and softer towards the top. Many smaller side branches shoot from the main stems. The stems are ribbed with strong swollen lines that run all the way up. The soft leaves are finely divided, almost fern-like. The upper surface of the leaves is dark green whereas the undersides and the stems are covered with small white hairs, which give the shrub the characteristic overall grey colour. *A. afra* flowers in late summer, from March to May<sup>19</sup>. It is used to cure diseases such as the common cold, diabetes mellitus, bronchial complaints and stomach disorders<sup>21</sup>. The main components of

*Artemisia afra* from the literature are 1,8-cineol and two ketones ( $\alpha$ - and  $\beta$ -) thujone, camphor and borneol<sup>21</sup>. Figure 3.1 shows the typical *Artemisia afra* plant.



**Figure 2.1** *Artemisia afra* plant<sup>20</sup>.

### 2.3.2 *Tagetes minuta* (kakiebos)

*Tagetes minuta* is an annual member of the *compositae* family. The plant grows to a height of 50-150 cm with a single stem highly branched at the top. It is of South American origin although it has been introduced to Europe, Australia and Eastern and Southern Africa. In the southern countries, *Tagetes minuta* oil has numerous applications, as an insect repellent and in treatment of certain illnesses such as smallpox, earache, colds and to reduce fevers. In addition, it has been recognised to possess hypotensive, spasmolytic, anti-inflammatory, antimicrobial and antifungal properties<sup>22</sup>. The main oil components such as dihydrotagetone, (Z)- and (E)- tagetones and (Z)- and (E)- tagetenones have interesting aroma properties. Inconsistency and diversity of the composition of *Tagetes minuta* oil has

been reported to depend highly on external factors such as place of harvest and different development stages of the plant during harvest<sup>21</sup>.

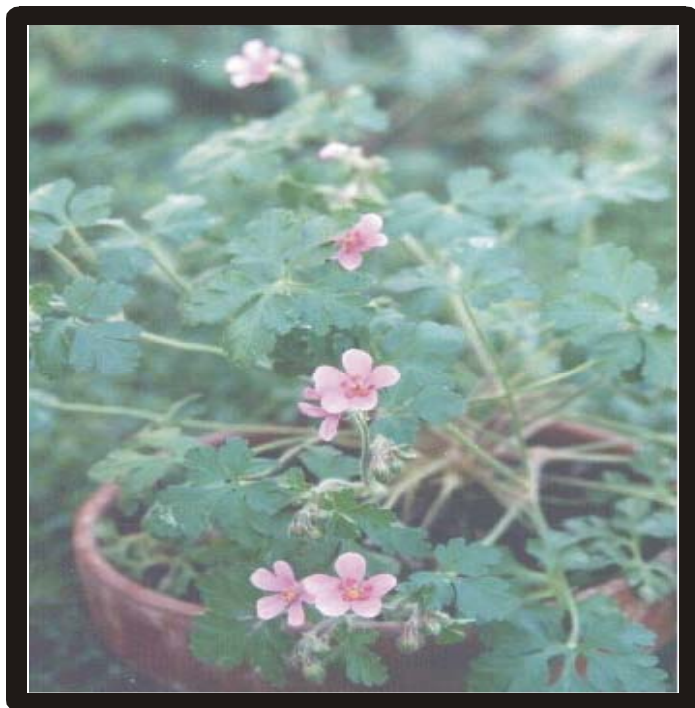


**Figure 2.2** *Tagetes minuta* plant<sup>20</sup>

### **2.3.3 *Pelargonium* (Geranium)**

More than 200 *Pelargonium* species have been identified. *Pelargonium radens* and *P. capitatum* are some of the *Pelargonium* species indigenous to South Africa. Hybrids of these species are cultivated from which the commercially important geranium oil is extracted. The aromatic plant is also cultivated for extraction of commercial rhodinol (mixture of linalool, citronellol and geraniol). Figure 2.3 shows a typical plant of pelargonium. Some of the main constituents found for pelargonium are  $\alpha$ -pinene,  $\alpha$ -phellandrene, p-cymene,  $\gamma$ -terpinene,  $\beta$ -caryophyllene, guaia-6,9-diene, germacrene D,  $\zeta$ -cadiene, citronellol, terpinen-4-ol, 10-epi- $\gamma$ -eudesmol, citronellyl formate and unidentified sesquiterpenes. The loss of oxygenated constituents in distillation has been observed:

which makes the aroma of the oil incomplete in terms of organoleptic richness and fullness<sup>23</sup>.



**Figure 2.3.** *Pelargonium capitatum* plant<sup>20</sup>

#### **2.3.4 *Cymbopogon* (lemongrass)**

Lemongrass, a perennial herb widely cultivated in the tropics and subtropics, designates two different species, *Cymbopogon flexuosus* and *C. citratus*. The plant grows in dense clumps and may reach diameters of up to 2 centimeters. The leaves may be up to 1 metre long. The plant needs a warm, humid climate and full sun. The quality of lemongrass oil is generally determined by the content of citral, the aldehyde responsible for the lemon odour<sup>24</sup>. Citral consists of the *cis*-isomer, geranial, and the *trans* isomer, neral. These two are normally present in a ratio of about 2 to 1. *Cymbopogon flexuosus* has a far higher citral content than *C. citratus*. Also *C. flexuosus* has a detectable amount of caryophyllene, which is absent in *C. citratus*<sup>25</sup>.



Some of the constituents of the essential oil are myrcene, citronellol, methyl heptanone, dipentene, geraniol, limonene, nerol and farnesol. Citral, extracted from the oil, is used in flavoring soft drinks, in scenting soaps and detergent, as a fragrance in perfumes and cosmetics, and as a mask for disagreeable odours in several industrial products<sup>5</sup>.



**Figure 2.4.** Lemongrass plant<sup>20</sup>.

## 2.4 Conclusion

The advantages of a particular isolation method of essential oils compared to others is closely related to the objectives of our study of the essential oils composition of given material. If the purpose is only to demonstrate the improvement of a particular technique of separation or sample injection, then the composition of the essential oil is of rather limited importance. However, if the intention is to study the true composition of the oil for purposes such as quality control or biosynthetic pathway of the oil, then the isolation technique plays an important role as shown by the discussions of the isolation methods for essential oils.

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# CHAPTER 3

## METHODS FOR ESSENTIAL OIL ANALYSIS

### 3.1 Introduction

When purchasing essential oil raw material, an industrial company has to pay careful attention to quality control. Sometimes, the essential oil composition is falsified by addition of cheaper oils (adulteration). Also the compositions of essential oils vary significantly with place of origin, harvest season, and weather<sup>1</sup>. The complex molecular composition of many essential oils demands high resolution separation to characterise the range of compounds making up the oils. In addition, it is often necessary to study small differences between oils that correspond to variation in geographic or genetic origin of plant material. Furthermore, it is important to study the active biological components that have some medicinal purpose. The analysis of essential oils can be summarized as follows<sup>2</sup>:

- The qualitative composition (search for new interesting components)
- The quantitative determination of trace components
- The detection of adulteration of true essential oils by dilution with cheaper oil or synthetic materials

Chromatography, especially gas chromatography (GC) and mass spectroscopy (MS) have been the most applied analytical techniques for essential oil analysis<sup>4</sup>. Because of the complexity of essential oil compositions, there has been a high demand for sophisticated instruments to analyse them. The limitation of GC and GC-MS has pushed chromatographers to dig deep in search of better methods to analyse essential oil volatiles such as improvement in sample preparation prior to injection, advanced sample injection methods: Solid-Phase Matrix Extraction (SPME)<sup>5</sup>, Headspace-GC<sup>6</sup> or coupling of analytical instruments to increase the separation power of one-dimensional techniques. This chapter discusses some of the developments in chromatography for essential oil analysis starting from gas chromatography to coupled techniques.

### 3.2 Essential oil analysis by gas chromatography

Chemical analysis as we know it today could not exist without gas chromatography, it dominates our analytical endeavours in fields as diverse as food, flavours and fragrance, petrochemicals, pharmaceutical and environmental studies. Capillary GC has strongly contributed to the development of the essential oil science from both the academic research and the industrial point of view (quality control, new sources for odoriferous compounds). In the early days, only metal columns were used, packed with a more or less inert support material coated with polar or apolar liquid stationary phases. The introduction of Golay columns<sup>7</sup> and further improvement by Martin<sup>8</sup>, who suggested decreasing the diameter of the column in order to increase the analysis sensitivity, thus enabling work on very small amounts of substances, made GC a powerful technique.

Many efforts have been made to improve separation in GC by working with columns of a very long length, with reduced diameter or selective stationary phases<sup>9</sup>. Sample preparation is one of the most important stages in gas chromatography analysis. Direct methods are available for analysis of volatiles that avoid solvent use. These are static headspace<sup>6</sup>, SPME<sup>5</sup>, and direct thermal desorption<sup>10</sup>. Analysis of extremely complex

samples, for example profiling the volatiles from natural products or essential oils requires adequate sampling and detection of compounds varying greatly in both boiling point and structure. In this case, a technique that accurately reflects the relative proportion of each analyte is preferred. A study was undertaken to compare the sensitivity of several sampling techniques (Static headspace, SPME and direct thermal desorption) for direct analysis of volatiles<sup>11</sup>.

Headspace analysis is a powerful tool for solving problems such as trace detection, or for checking a wide variety of different materials<sup>12</sup>. It is one of the most useful auxiliary techniques available in gas chromatography. It is important to consider that the GC analysis of essential oils is commonly carried out to evaluate a particular plant or herb for its percentage oil content and constituents. The analytical values obtained in this way do not, however, refer directly to the volatile substances present in the herb itself and may not truly reflect the real quality of herb, depending on the isolation technique used. However, application of a method such as static headspace in GC, improves the sensitivity and allows detection of lower concentration compounds<sup>11</sup>.

The advantages of using SPME include the need for much less plant material, more rapid and reproducible sample preparation, less opportunity for oxidation of volatiles to occur, and no need for the use of organic solvents. A method was developed to determine the concentration of limonene oxide in essential oils and beverages using SPME<sup>13</sup>. A headspace sampling technique was used to reduce or eliminate the presence of less volatile components. Bicchi *et al.* described their work on the effect of fibre coating on headspace SPME from aroma and medicinal plants<sup>14</sup>. As an example, the comparison of static headspace GC (HS-GC) with headspace GC using different polymeric coatings was presented. Several different SPME fibers were used, varying in polymer thickness, polymer cross-linking and bonding, and polarity of the polymer<sup>14</sup>. For each fiber tested, the sampling time is optimized for reproducible results. The 7- $\mu$ m-thick bonded polydimethylsiloxane (PDMS) fiber provided the best results. External standards were used for quantitation of the sample composition.

Another interesting development of GC methods in essential oil analysis is the application of the GC-olfactometry (odour evaluation) or GC-sniffing technique<sup>15</sup>. In the essential oil GC-sniffing method, the analyst writes notes on the GC chromatogram when odour is perceived by his nose as detector. In this way, it is possible to point out peaks and regions in the chromatogram where odours are detected. The method is also limited because further work is required to identify the peaks for example by GC-MS.

All these developments have contributed much to improve the separation power of GC in terms of number of peaks separated, as well as qualitative and quantitative information generated. The combination of GC-olfactometry to correlate the single aroma impression from the GC eluate with gas chromatographic (retention times, Kovats indices) and mass spectrometric data has been very successful in essential oil analysis. The technology of high speed or fast GC has been introduced lately in the market and its application to essential oils is limited as compared to the old fashioned capillary GC method. The following section discusses the requirements for fast GC and some of its applications in essential oils analysis.

### **3.2.1 High-speed gas chromatography**

Over the past few years, instruments and methods were developed to dramatically increase the analysis speed of capillary GC. According to Blumberg and Klee<sup>16</sup> fast capillary gas chromatography should have an average peak width less than one second. They assign a "super fast GC" when the average peak width is around 100 ms and "Ultra fast GC" when the peak width is less than 10 ms. Table 3.3 depicts the ranges of conditions corresponding to conventional, fast and ultra fast GC in terms of analysis time, heating rates, column length, internal diameter and peak width.

**Table 3.3.** Schematic representation of common requirements for reaching a high speed of analysis in GC<sup>16</sup>

<b>Description</b>	<b>Heating rate (°C/min)</b>	<b>Column length (m)</b>	<b>Column i.d. (µm)</b>	<b>Analysis time (min)</b>	<b>Peak Width (s)</b>
<b>Ultra Fast</b>	60 - 1200	5 – 2	100 - 50	~ 1	0.2 – 0.05
<b>Fast</b>	20 - 60	15 – 5	250 - 100	~ 10	5 - 0.5
<b>Conventional</b>	1 - 20	60 - 15	320 – 250	~ 30	10 – 5

The most important parameters that influence the speed of a GC analysis are the length and diameter of the GC column, velocity of the mobile phase and oven temperature programming rates. A significant reduction in analysis time may be achieved. Loss in resolution can be partly offset by using narrow-bore columns when using short columns combined with fast temperature programming. These columns have rather a low sample capacity and require high inlet pressure, narrow injection band width and a fast detector to record the (narrow) peak profiles properly<sup>17</sup>. The results obtained so far are very promising in the commercial routine laboratories.

Temperature is one of the crucial parameters determining the time required for separation of chromatographic zones<sup>18</sup>. Changing the temperature of the chromatographic column can dramatically change the retention of substances on the sorbent layer. The most important benefit of temperature programming in gas chromatography is a substantial reduction of analysis time. The maximum rate at which the oven temperature linearly increases is related to the thermal mass of the oven cavity, the column, the heater power, the efficiency of the oven wall insulation and the



differential temperature between the inner part of the oven and its external environment<sup>19</sup>. A conventional oven is relatively large and the heating process is much slower and cooling times are often very long.

Although selectivity parameters such as small internal diameters, shorter column lengths, thinner films, faster flow rates, higher pressure, vacuum at the outlet reduces the analysis time in GC<sup>18</sup>, the temperature remains vital, since it may significantly reduce the analysis time for separation of mixtures containing a wide range of boiling points. Two approaches to achieve faster gas chromatographic separations are<sup>16</sup> :

1. Fast temperature programming with conventional ovens and
2. Fast temperature programming by column resistive heating.

The temperature has a profound effect on analysis time, as a result, considerable time could be saved by better optimization of the program conditions. Fast temperature programming with conventional ovens combines optimized conditions of microbore columns and thin film stationary phases with powerful oven heaters<sup>19</sup>. Ramp rates have increased from 1 – 2 °C/sec to 150 °C/sec<sup>19</sup>. The ramp rate is limited due to high temperature gradients occurring across the oven volume.

The possibility to achieve fast heating and cooling rates of the analytical column is a recent commercial venture in speeding up the analysis time whilst maintaining good resolution. Fast temperature-programmed gas chromatography with a resistive heating technique was first proposed by Dal Nogare<sup>20</sup> in 1958 and further reaffirmed by Rounbehler in 1991<sup>21</sup>. Numerous resistively heated systems have recently been developed to allow faster temperature programming and cooling times. They can achieve heating rates of 20 °C/sec (1200 °C/min) and cool down very rapidly from 300 °C to 50 °C in less than 1 minute<sup>16</sup>.

Apart from the fast cooling and heating rates the instrumental capability of detecting fast signals with the best peak fidelity and peak widths in the order of 100 ms or even

less, is one of the requirements for high speed GC techniques<sup>22</sup>. The detector response time should always be a minor contributing factor in the overall peak variance. The detector time-constant has to be compatible with the peak width otherwise peak distortion will occur with consequent loss of resolution<sup>23</sup>. A fast electrometer board has been developed for the flame ionization detector<sup>22</sup> to increase the detection response speed of the FID.

Hail and Yost<sup>24</sup> used resistively heated aluminium clad capillary columns. Overton and co-workers<sup>25</sup> have described a direct resistive heating system incorporating heating and temperature sensing elements placed along the column. The approach was utilized for heating a very short 1 m to 2 m narrow bore column mounted in a portable fast GC. Though Overton's development represented an important step ahead by strongly reducing the instrument overall electric power consumption, their system had the limitations of only being applicable to very short columns and only reached a maximum operating temperature of 250 °C.

Other researchers also heated aluminium-coated fused-silica columns directly by passing a current through the metallic outer layer<sup>26,27</sup>. The device had very fast heating and cooling rates, but was hindered by two problems namely, the outer metallic coating was uneven leading to unwanted temperature gradients, and secondly, each column coil had to be carefully insulated, thermally and electrically from adjacent coils and the column supports. Any contact would result in a large thermal gradient and short-circuit. One of the limitations of the direct resistive heating method is the large electric resistance of a long capillary<sup>24</sup>. A very short column with a length of 1-2 m is rational to use. The problem with longer columns includes lack of uniform heating, poor precision in temperature control and interference from environmental temperature fluctuation.

### 3.2.2 Fast gas chromatography in essential oils analysis

The applicability of ultra fast GC in essential oil analysis as compared to conventional GC is limited. A comparative study of ultra-fast GC with resistively heated column and conventional GC with reduced inner diameter column (0.25 mm) of different length (5 and 25 m long) was undertaken with different essential oils<sup>28</sup>. Essential oils studied were of different complexities (Chamomile, peppermint, rosemary and sage). Comparable results of major peaks expected in these oils were observed for both GC and ultra-fast GC methods. Although ultra-fast GC has emerged as a powerful GC method to provide information in a very short time, resolution is compromised by rapid heating and high flow rates in a short column. Because of the limited peak capacity in fast GC more of the peaks co-elute.

The coupling of fast resistively heated GC to another technique can utilize full capabilities of the method. In this thesis, fast resistively heated GC is coupled to SFC where the problem of co-elution in fast GC alone is reduced by analysis of presented SFC fractions rather than the entire sample simultaneously. Another interesting work in high-speed GC, was the approach of using series coupled capillary columns with a pressure switch technique and fast oven temperature programming<sup>29</sup>. Analysis times of less than 2.5 min were obtained. Although short analysis times were obtained peak co-elution was still a problem. Operating at a spectral acquisition rate of 25 spectra / second was used to deconvolute overlapping peaks<sup>29</sup>.

The goal of chromatography has always been to obtain a complete separation of components in a sample. However, application of one-dimensional techniques (the use of conventional GC with long column lengths or fast GC) has shown some limitations. Multidimensional chromatography has emerged in the late 80's to provide solutions to the problem of peak overlap. The following section discusses the principal ideas of multidimensional chromatography and its application to essential oils analysis.

### 3.3 Multidimensional Chromatography

#### 3.3.1 Concepts in Multidimensional separation

Multidimensional chromatography is defined as the chromatographic process where two or more analytical techniques are coupled together to enhance their separation power<sup>30</sup>. The combination can be either two chromatographic techniques or a chromatographic technique with spectroscopy.

The ultimate goal of chromatography has been to effectively separate sample mixtures with the largest number of compounds in the shortest time. For samples with limited complexity, the classical approach has always been the search for chromatographic systems or phases with optimal selectivity. The limit of this approach is being reached very quickly. Improvements in chromatographic efficiency, or the number of compounds that can be separated in a single chromatographic run, has received special attention with columns of ever reducing radius or particle size becoming available commercially.

An estimate of the separating power of a single chromatographic system can be obtained by considering the maximum number of components that can be placed into the available separation space with a given resolution that satisfies the analytical goals. Giddings<sup>31</sup> introduced the concept of peak capacity ( $n_c$ ) to measure the resolving power of the chromatographic system. Peak capacity is defined as the number of compounds that can be placed side-by-side in a separation space with a given resolution and is given by the following equation:

$$n_c = \left(1 + \frac{N^{1/2}}{r}\right) \ln(1 + K'_i) \quad [3-1]$$

where  $N$  is the number of theoretical plates,  $r$  the standard deviation taken as equaling the peak width and  $K'_i$ , the capacity factor of the last peak in a series.

The need to analyse samples of increasing complexity and at lower detection limits has placed more requirements that are stringent on the separating power required for analysis. A single chromatographic system is inadequate to handle very complex samples and attempts at analysing complex samples often lead to long analysis times. The analysis of complex samples often requires the use of many separation mechanisms to reduce peak overlap and to obtain information on individual components<sup>32,33</sup>.

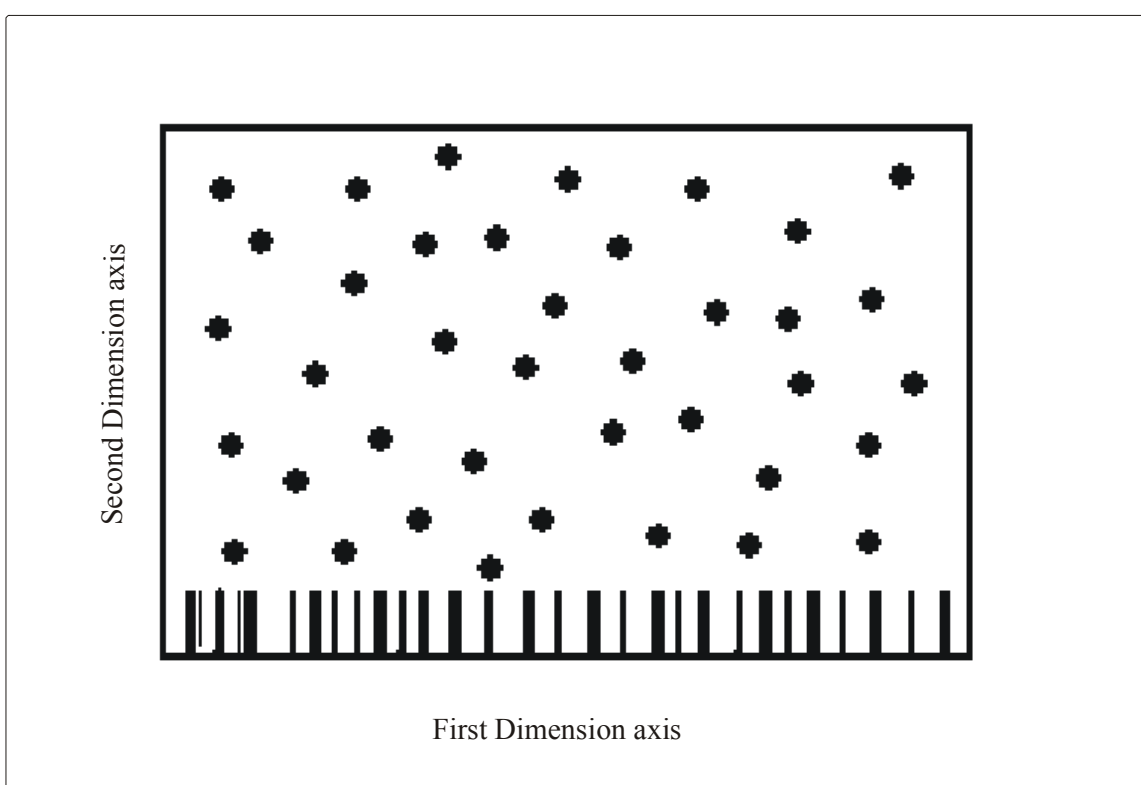
Mass selective detectors that are capable of deconvoluting merged peaks have been employed to aid the separating powers of single chromatographic systems and for positive identification of compounds<sup>30</sup>. However, selective detection is only successful when different responses are produced for the individual compounds represented by the merged peaks. An alternative solution appeared with the emergence of the special concept in separation science, known as Comprehensive Multidimensional Separations or Comprehensive Multidimensional Chromatography<sup>34</sup>.

The degree of information loss due to encroaching peak overlap or peak crowding in the analysis of complex mixtures was the driving force for the original development of comprehensive multidimensional chromatographic separation techniques. In order for two techniques to be successfully coupled, several criteria need to be addressed. Giddings<sup>30</sup> defined two vital criteria that need to be considered for comprehensive multidimensional systems:

- 1. Components must be subjected to two or more largely independent (separating techniques).*
- 2. The separation must be structured in such a way, that whenever two components are adequately separated in one displacement step, they must remain resolved throughout the process.*

In general, multidimensional systems provide more separating space than one-dimensional systems, allowing component peaks to spread out across additional coordinates, thus reducing peaks overlap. Figure 3-1 shows how component peaks,

represented by spots, are distributed randomly over a two-dimensional plane, which has sufficient space to minimize the overlap of individual peaks because of the additional dimension. When all these components are compressed onto a single axis, as represented by lines on the first dimension, they are very crowded and eventually overlap one another due to band broadening<sup>35</sup>. In this way most of the information will be lost because low concentration compounds will be hidden within high concentration component peaks that dominate the chromatogram.



**Figure 3.1.** A two-dimensional plane representation of a complex sample, separated using two independent separation methods<sup>35</sup> (depicted by the stars) and a representation of the same sample separated using only one separation axis (Represented by the lines).

A multidimensional separation generates higher peak capacity only when the retention mechanisms in the constituent dimensions are independent of each other. When there is correlation in the retention mechanisms across separation dimensions, the peak capacity

is reduced to some fraction of that theoretically available. Any correlation between the selectivity mechanisms of the two separations leads to the wasteful production of separation space offered by multidimensional separation that cannot be used<sup>36</sup>. An information theory analysis showed that the information content of a multidimensional system is the sum of the mean information content of each individual dimension minus the cross information<sup>37</sup>.

Minimizing the cross-information or synentropy in a multidimensional separation is very important. When synentropy is large, much of the separation space is unoccupied or is completely inaccessible and the sample components tend to cluster along a diagonal. Minimising synentropy will maximise the efficiency of the information generated by multidimensional separations. However, in an orthogonal multidimensional separation, constituent dimensions are operated independently and the usable peak capacity equals the product of the peak capacities of the constituent dimensions<sup>38</sup>. It has been shown that peak capacity is in practice less than the product of the two dimensions of comprehensive-multidimensional chromatography<sup>39</sup>. Also that an order of magnitude improvement in peak capacity is expected from a GCxGC analysis as compared to one-dimensional GC<sup>40</sup>.

The accurate description of the chromatographic processes leading to the separation of individual compounds in complex mixtures is difficult. Many different theories have been developed in an attempt to describe the overcrowding of compounds in chromatograms and to find solutions to the problem. Giddings' statistical model of component overlap (SMO) theory stands out as the most easily understood approach to quantitatively describe the consequences of statistical events in chromatographic processes<sup>41</sup>. The concept can be readily understood even though its detailed mathematical treatment is quite complex. Simple SMO theory explains the fundamental causes of peak overlap and suggest solutions to the problem.

With this model the approximate number of visible peaks,  $P$  is given by:

$$P = me^{-m/n_c} \quad [3-2]$$

where  $P$  is the number of peaks appearing as singlets,  $m$  reflects the number of compounds in the chromatogram and  $n_c$  the peak capacity of the column. The number of single-component peaks,  $S$ , which can be expected is given by :

$$S = me^{\frac{-m}{n}} = me^{-2\alpha} \quad [3-3]$$

where  $\alpha$  is the ratio between the number of analytes in a sample and the available space.

The statistical model of overlap indicates that the number of peaks per second can be estimated when two parameters are known: The peak capacity and the number of components,  $m$ . Although it is difficult to estimate the value of  $m$  for complex mixtures, SMO theory provides a methodology for estimating  $m$ <sup>33,41</sup>. The shortcomings of the original SMO theory is that it does not make adequate provision for distorted peaks, peaks of largely different sizes and chromatographic disturbances, including noise<sup>42</sup>.

Although multidimensional separation can create very high peak capacities, the success of enhancing peak capacity in resolving a particular complex sample mixture depends strongly on whether the distribution of component peaks in the chromatogram is ordered or disordered. A disordered distribution in a chromatogram results from components that come from a number of chemical families. Peak crowding is common in disordered distributions even with a very high  $n_c$ , therefore it is important to understand the origin of peak order/disorder in multidimensional separations to address



the levels of peak distribution and thus separation efficiency, by choosing the correct combination of separation mechanisms.

Giddings<sup>35</sup>, observed that the underlying difference between ordered and disordered distributions of component peaks in separation systems is related to sample complexity as measured by a newly defined parameter, the sample dimensionality,  $S$ . Sample dimensionality,  $S$ , is defined as the number of independent variables that must be specified to identify the components of the sample mixture.

Sample dimensionality in conjunction with system dimensionality  $n$  can provide some predictive capabilities with regard to ordered versus disordered component distribution following multidimensional separation. It also indicates where the full peak capacity of a multidimensional system can be substantially exploited. When the dimensionality of the sample exceeds that of the system, components of the sample will not be systematically resolved in the system. The resulting retention (separation) pattern is disordered<sup>35</sup>. When only some of the sample dimensions are of interest it may be required to analyze a sample along a selected few of the possible sample dimensions only.  $S''$  is the required dimensionality defined as the number of variables that must be determined for the purpose of the analysis. In an ideal system all other sample properties are to be weakly expressed and in such cases  $S''=S'$ , where  $S'$  is the apparent dimensionality of  $n = S''$ <sup>35</sup>. Then the sample can be systemically analysed in a system of given dimensionality. Apparent dimensionality is equal to the number of variables expressed strongly enough to produce suitable resolution<sup>35</sup>.

In chapter 4 only separation along the dimension of polarity is required and experimental conditions need to be adjusted to ensure that the other dimensions such as volatility are weakly expressed. This type of analysis is generally known as group-type or chemical class separation<sup>42</sup>. The concept is further explored in Chapter 5 where the separation is no longer dependent along one dimension (polarity) only, but additional separation is affected by a second dimension (volatility). The resulting

multidimensional chromatogram is an ordered one with independent polarity and volatility dimensions.

### **3.3.2 Peak capacity enhancement**

The accidental overlap of peaks represents a serious problem for most chemically complex samples, no matter what separation systems are used<sup>30</sup>. Numerous, approaches can be employed to enhance the peak capacity of a chromatographic separation. The most common approach to improve peak capacity is to increase column length, decrease column diameter, or a combination of both. However, this approach can be very costly in analysis time and detection limit when chromatography of a truly complex sample is carried out on a single column. Increasing the chromatographic column length does not substantially enhance the peak capacity, because peak capacity only increases with the square root of the column length while the retention and column back-pressure increase linearly with the length. The result is longer analysis times, broader peaks and consequently worse limits of detection<sup>45</sup>. A novel way to increase column length and increase the peak capacity has been obtained by moving the sample through the same column in a repetitive fashion and the process is referred to as effluent recycling<sup>41</sup>. One of the benefits of multidimensional systems is that they provide an alternative solution to peak capacity enhancement that results in a remarkable improvement in the physical separation of individual compounds.

### **3.3.3 Comprehensive multidimensional chromatography**

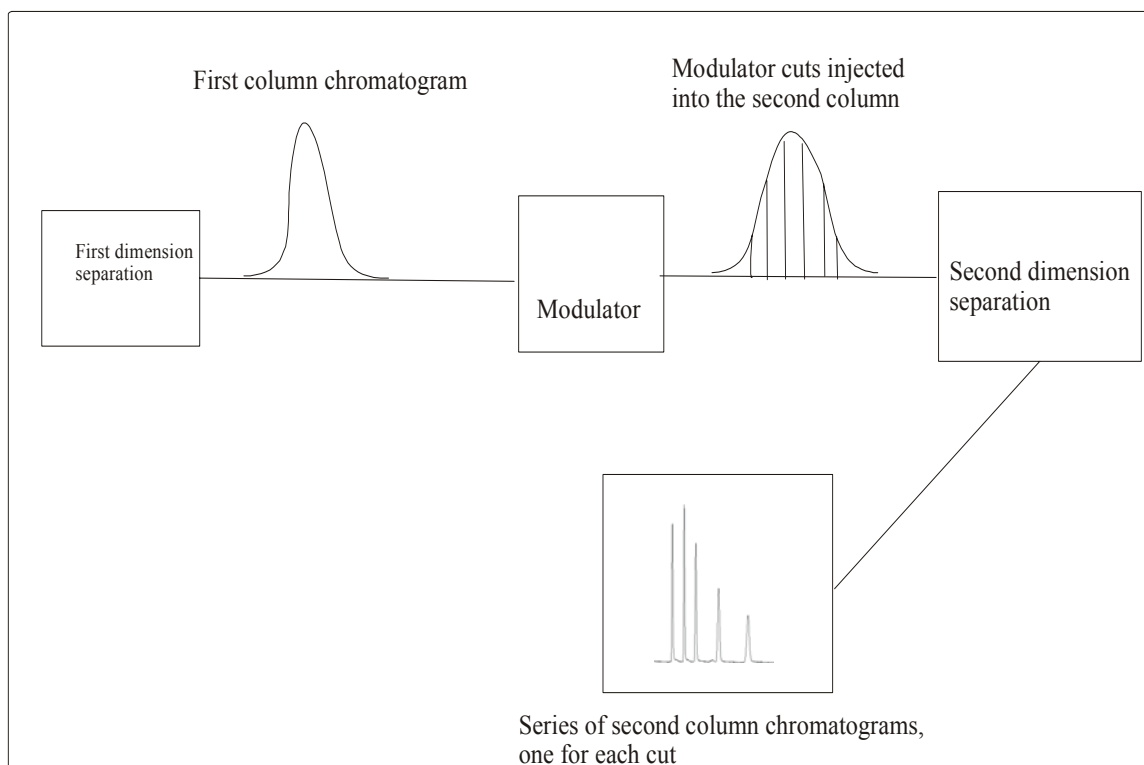
Multidimensional chromatography with coupled columns usually requires heart-cutting techniques (switching valves or trapping devices) to transfer selected portions of the eluents from the first column to the second column<sup>30</sup>. These techniques can provide superb resolution for the selected portions; however, most of the analytical range is sacrificed because most of the sample from the first separation is not allowed to pass to

the second column for further separation<sup>43</sup>. Such two-dimensional chromatography is regarded as non-comprehensive.

Repeatedly cutting small sections of the first chromatogram and subjecting them sequentially to a second separation without sample discrimination or loss of resolution on all eluted peaks from the first column is known as **comprehensive multidimensional chromatography (CMC)**. Technically it is an extension of the heart-cutting method. It provides a new separation technique operating independently in more than one dimension to produce orthogonal separations<sup>44</sup>.

Figure 2.2 shows the process of comprehensive two-dimensional elution chromatography. The process normally involve three basic steps: A first dimension separation, followed by accumulation of a small section of the eluent from the first dimension by the modulator and re-injection into a secondary column for the second stage of the separation. The resulting second dimension chromatograms represents data points on the first chromatogram.

A comprehensive multidimensional separation must be structured such that whenever two components are adequately resolved in the first dimension separation step, they must generally remain resolved throughout the process<sup>30</sup>. This requirement is complicated by the operational aspects of switching effectively from one separation step to another and by data acquisition and interpretation problems. The process of solute focusing or re-injection of the effluent from the primary column into the secondary column has to be reproducible and non-discriminatory with regard to the analyte properties.



**Figure 3.2** Representation of the steps involved in comprehensive two-dimensional chromatography.

### 3.3.3.1 LCxLC

Two-Dimensional liquid chromatography was first demonstrated by Erni and Frei<sup>37</sup>. The system involved a heart-cutting interface coupling the two liquid chromatographs, however, their data structure was not comprehensive in nature. More improvements were needed for the system to meet requirements of a comprehensive separation process. Their data structure being considered showed correlation between the retention mechanisms of the two separations which lead to the wasteful production of separation space that was not used. This ruled out their system data structure from being considered to be comprehensive.

Based on the knowledge gained from the two-dimensional liquid chromatography developed by Erni and Frei, Bushey and Jorgenson<sup>45</sup> built an automated comprehensive

two-dimensional liquid chromatograph (LCxLC). In the system a stopped-flow operation or off-line technique was not necessary. The separation speed in each dimension was adjusted such that the entire effluent from the first column was introduced into the second column without directing any portion to waste. They used as the first column a microbore cation-exchange column under gradient conditions. This was coupled through an eight-port switching valve to a size-exclusion column.

The combination of cation-exchange and size-exclusion mechanisms are orthogonal to provide complementary information. The improved resolving power of the system was best demonstrated by the separation of protein samples hemoglobin from P-lactoglobulin A and ribonuclease A<sup>45</sup>.

### 3.3.3.2 GCxGC

A two dimensional separation system, which met the comprehensive two-dimensional requirements, was first proposed by Guiochon and co-workers<sup>46</sup> in the early 1980s. Though the system possessed some features of comprehensive multidimensional chromatography, more development of the system was needed. Liu and Phillips<sup>47</sup>, pioneered the modern true comprehensive two-dimensional Gas Chromatograph (GCxGC) using an on-column thermal modulator interface to couple the two separation mechanisms. Fast sample transfer between columns was achieved using an on-column thermal modulator, which was an effective sample introduction device in fast GC.

In GCxGC systems employing a thermal modulation interface, the carrier gas containing compounds from the first column continuously enters the second column. The temperature of the first few centimetres of the modulator column is modulated by an electric current pulse train applied to a thin electrically conductive film on the outside of the column. During the relatively cool part of the modulation cycle, compounds accumulate within the modulator portion of the column. The temperature of the modulator is rapidly raised by the electrical current pulse, releasing the sample from

the stationary phase, and refocusing it into the second column as a sharp concentrated pulse<sup>47</sup>. The modulator is built on-column, no extra column band broadening occurs and the resulting concentrated pulse is automatically of an appropriate volume and duration for injection into the secondary column. An alternative way to effect stationary phase focusing was developed by Marriot and Kinghorn<sup>48,49,50</sup>. They used a moving cryogenic trap to focus analytes on the second column. Using carbon dioxide as cryogen, analytes as volatile as hexane can successfully be immobilized. All peaks eluting from the first column are chopped into a series of high-speed injection pulses by the modulator (**Figure 3.2**) and transferred into the second column. Ideally analysis times in the second dimension are short enough to effect at least five chromatograms during peak duration in the first column. As a result, peaks resolved by the first column do not re-merge during sample transfer between dimensions.

Identification of unknown compounds is difficult with single-column GC, because retention is caused by a combination of volatility and polarity separation mechanisms<sup>51</sup>. The location of the analyte peak in a GCxGC chromatogram serves as a powerful selective identification tool. With the GCxGC system the two retention times can be used to measure the polarity and volatility of a substance. GCxGC is similar to gas chromatography – mass spectrometry (GC-MS) in the sense that both methods combine independent analytical techniques and generate comprehensive two-dimensional data. Each data point on the first chromatogram is represented by a mass scan effected by the mass spectrometer in the case of GC-MS or a second fast chromatogram in the case of GCxGC.

The range of samples that can be analyzed with GCxGC is restricted by the upper temperature limit of the polar column in the second dimension. The maximum boiling point of an analyzable sample is limited to 400°C<sup>52</sup>. When the thermal modulator is used, the final boiling points of samples are restricted by the fact that the modulator tube (front part of the polar column) should be warmer than the rest of the second column to ensure sharp injection bandwidths.

### **3.3.3.3 SFCxGC**

The first comprehensive two-dimensional supercritical fluid chromatography and gas chromatography (SFCxGC) system was demonstrated by Lee and co-workers<sup>53</sup> using a thermal desorption modulator as the interface between the SFC and GC columns. The first dimension achieves molecular shape analysis (SFC). This is followed in the second dimension by volatility analysis (GC). An integral flow restrictor was installed at the head of the GC column and an on-column thermal desorption modulator was placed after the flow restrictor. Both columns of the SFC and GC were temperature programmed at the same rate and operated at the same temperature. CO<sub>2</sub> was the carrier gas in both dimensions.

Using thermal desorption as the modulator interface in comprehensive two-dimensional SFCxGC poses some limitations on the application range of the technique, given that thermal desorption can not be used for thermally labile compounds. For analysis of large molecules it is difficult to use thermal desorption without damaging the stationary phase<sup>54</sup>. Development of other types of interfaces, or the use of high temperature stationary phases in the modulator, along with independent temperature control in each dimension would improve the application range of the technique.

### **3.3.3.4 SFCxGC with independent temperature programming in the second Dimension.**

A comprehensive two-dimensional supercritical fluid and independent temperature-programmed gas chromatograph (SFCxGC) instrument has been designed and constructed in our laboratory<sup>42</sup>. The technique employs supercritical fluid chromatography to effect group type separation. This is coupled on-line, through a modulating device, to a resistively heated, fast temperature programmed gas chromatograph for volatility analysis. In SFCxGC, arrangement of the separation mechanisms is the reverse of GCxGC<sup>47</sup>, where volatility separation in the first dimension normally precedes polar separation in the second dimension.

This SFCxGC is different to the previously demonstrated SFCxGC<sup>53</sup> instrumentation, where a standard GCxGC<sup>47</sup> thermal modulator was used. The SFCxGC instrumentation demonstrated in this thesis, is equipped with a modulation device employing pressure drop modulation as well as stop flow chromatography<sup>42</sup>. This is a low temperature modulation technique that is gentle with thermally labile compounds. Furthermore, the SFC column was operated isothermally at a low temperature to make the most of the separation capabilities offered by SFC. It has been shown that selectivity based on polarity, enantiomeric, size or shape differences are temperature sensitive<sup>55</sup>. SFCxGC, as demonstrated by Lee, did not make the most of this selectivity advantage offered by SFC as both SFC and GC columns were heated simultaneously<sup>54</sup>.

The second dimension in SFCxGC is a fast temperature programmed GC using resistive heating of a capillary column. A stainless steel capillary column was used where the column is its own heating element<sup>42</sup>. A 30V power supply was used to supply a current to the column. A thermocouple was mounted in direct contact with the column to measure the temperature. The temperature gradient heating of the capillary column was controlled through a program running from a computer using a Proportional Integral Derivative (PID) algorithm for feedback control.

This instrumentation was previously applied to the analysis of petrochemical samples<sup>42</sup>. In this thesis the same instrumentation is applied to the analysis of essential oils. Various essential oils produced in South Africa were analysed with the aim of showing the capabilities of the SFCxGC instrument to qualitatively differentiate oil samples of the same species but obtained in different geographical location and distinguish two oils of the same species.



### 3.4 Coupled techniques in essential oil analysis

#### 3.4.1 Gas chromatography and Mass Spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) has probably been the most effective multidimensional method for analysis and identification of essential oils<sup>56</sup>. In many cases the mass spectra of essential oil components are unfortunately very similar. Thus, within the broad class of monoterpenes, a large number of isomers of the same molecular formula (but with different structure) exist and their mass spectra often bear close resemblance.

Since complex essential oils may have multiple overlapping peaks there will always be uncertainty regarding the purity of any recorded peak should the GC be used alone. The mass spectrometer may recognise overlap and apportion relative amounts to overlapping components. The availability of the accurate mass (high-resolution) MS is a valuable tool for confirmation of the molecular formula of detected unknown components<sup>57</sup>. With reference to essential oils, and in particular monoterpene hydrocarbons, accurate retention times are often of greater importance due to the mass spectral similarity of their isomers<sup>58</sup>.

One of the newer methods that has been proposed to give improved analysis of complex mixtures, especially for deconvolution of overlapping GC peaks, is known as time-of-flight mass spectrometry coupled to fast GC (GC-TOF-MS)<sup>59</sup>. TOF-MS has the capability of generating instantaneous spectra. Due to this fact, there is no bias occurring from the mismatch between scan rates and the peak abundance changes in the ion source (mass spectral “skewing”). Unlike quadropole mass spectrometers, TOF mass spectrometers provide uniform mass spectra across GC peaks, even for the narrow ones found in fast GC<sup>58</sup>.

Recently, better separations of essential oils were reported with GC-TOF-MS<sup>59,60</sup>. In TOF-MS fast spectral acquisition capabilities of 100+ mass spectra /second that are

compatible with fast GC peaks have recently attracted much attention from researchers and routine laboratories<sup>58</sup>.

### 3.4.2 Multidimensional GC and GCxGC

The application of multidimensional gas chromatography (MDGC) to essential oil analysis has been one of the most effective adopted technologies because of the improved resolving power the technique can offer when analysing complex mixtures. By effecting a heart-cut event of the analyte on a chosen region of a chromatogram (from 1<sup>st</sup> dimension), the desired components are transferred into a second, more selective column, where components are better resolved.

The role of MDGC is clearly to target a certain poorly separated region and provide increased resolution<sup>30</sup>. Quantitation or identification of components is significantly improved through the use of this method. This may be important for essential oils and specific components whose relative abundance may be required to study a particular aspect of the sample quality, history, source and biogenesis.

Though MDGC offers better separation, much of the information is lost because it heart-cuts only selected portions of the effluent from the first column. Some of the MDGC applications to specific components for fragrance analysis, such as analysis of enantiomeric constituents of cold-pressed and distilled fruits, have been reported<sup>61,62</sup>.

The recently described technique of comprehensive two-dimensional gas chromatography (GCxGC)<sup>47</sup> addresses a number of shortcomings of conventional MDGC when analysing very complex samples such as essential oils. So far there are only limited reports of GCxGC application to essential oils analysis compared to MDGC. Marriott and co-workers<sup>63</sup> used GCxGC to characterise and compare the tea tree and lavender oils. A coupled column combination of non-polar (5% phenyl equivalent) and polyethylene glycol phase columns were used to attain the desired

resolution. Dimandja and co-workers<sup>64</sup> reported work on the qualitative analysis of essential oils of peppermint (*Mentha piperita*) and spearmint (*Mentha spicata*) oils using GCxGC. High resolution GC-MS and linear retention indices (LRI) results for the lavender oil samples were compared with GCxGC results and many compounds could be identified, even without MS<sup>65</sup>.

The task of enantioselective analysis of essential oils is very challenging. The analysis of enantiomeric compositions of number of monoterpene hydrocarbons and oxygenated monoterpenes in the Australian tea tree (*Malaleuca alternifolia*) by GCxGC was reported<sup>66</sup>. GCxGC as a new technique has a promising future in the analysis of essential oils and enantiomeric composition of the oils.

### 3.4.3 High performance liquid and gas chromatography (HPLC-GC)

HPLC-GC (where the HPLC is coupled on-line to a GC) has been recognised as a separation technique providing a lot of information in a single run. A compiled review of coupled HPLC-GC in food and essential oil analysis is available<sup>67</sup>. The HPLC step achieves isolation of components of similar chemical group, primarily based on their polarity and as a result oxygenates will be separated from the saturated and unsaturated /aromatic hydrocarbons.

Numerous papers report the application of HPLC-GC-MS to bergamot oil<sup>68</sup>, the analysis of a wide range of oils<sup>69</sup> and also for chiral analysis<sup>70</sup>. Better results were obtained for the essential oils due to the efficient separation and reduced interference from overlapping peaks in the final GC. HPLC is a powerful technique that may be employed to obtain group separation, however, its interface to GC is hampered by problems normally associated with the removal of large volumes of HPLC eluent when introduced into the GC injection port.

### 3.4.3 Supercritical Fluid and Gas Chromatography (SFC-GC)

SFC is a separation technique that is often a compromise between GC and HPLC. SFC combines the group separation capabilities of HPLC with easy quantitation through the use of CO<sub>2</sub> mobile phase that is compatible with the universal flame ionization GC detector. Yamauchi and Saito<sup>71</sup> used semi-preparative scale packed-column SFC to separate lemon-peel oil into different chemical classes.

An SFC/GC system was used for group-type analysis of citrus essential oil<sup>72</sup>. The lemon-peel oil was fractionated on a silica-gel column into several compound types, namely, hydrocarbons, alcohols, aldehydes, esters and others using a stepwise pressure gradient and modifier added to the pure CO<sub>2</sub>. The eluted groups from SFC were re-injected into a GC for further separation of individual compounds.

### 3.5 Conclusion

A review of the results achieved over the last two decades show that gas chromatography has a the most important role in the study of essential oils, either on its own or coupled to mass spectrometry. The problem of peak overlap has posed serious problems in analysing samples of high complexity. Retention indices in combination with MS library data can identify and confirm oil composition only in ideal cases when peak overlap is limited. The recent development of ultra fast GC with resistively heated GC columns has only worsened the situation due to limited peak capacity. However the combination of ultra-fast GC in multidimensional chromatography can reduce peak overlap with enhanced peak capacity. A recent development in multidimensional technology, comprehensive multidimensional chromatography, has shown the power to separate complex mixtures in a very effective manner.

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# CHAPTER 4

## SUPERCRITICAL FLUID CHROMATOGRAPHY

### 4.1 Introduction

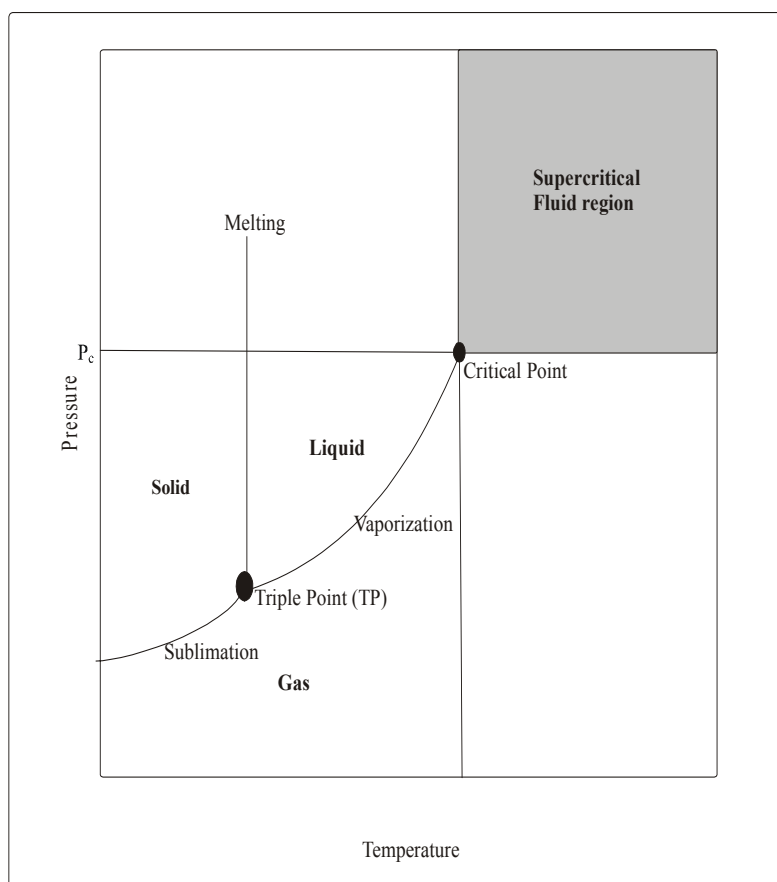
Chromatographic separation of highly complex samples is often impossible using single columns in a one-step separation process. The same problem applies to essential oil analysis. The complex molecular composition of many essential oils demand that high-resolution separation techniques be used to characterise the range of compounds making up the oils. Since essential oils are composed of different chemical classes such as terpene hydrocarbons, esters, ketones, aldehydes and alcohols, it will be advantageous to employ a separation technique that has capabilities of chemical class separation prior to high-resolution analysis. SFC can be used to offer improved group separation of complex mixtures of compounds compared to liquid chromatography on normal phase columns. The separation of complex sample mixtures such as essential oils into different chemical classes is one of the best methods to reduce the sample complexity. In Chapter 5 and 6 of this thesis, group-type separation of essential oils by SFC is reported.

## 4.2 Separation with supercritical fluids

### 4.2.1 Definition

A substance is said to be in the supercritical fluid state when heated above its critical temperature and compressed above its critical pressure and is referred to as a supercritical fluid. This can be seen as a very dense gas with a solvent strength comparable to that of liquids.

Figure 4.1 is a typical phase diagram for a pure substance that shows the temperature and pressure region where the substance occurs as a single phase (solid, liquid or gas)<sup>1</sup>. Three curves describe sublimation, melting and vaporization processes. They intersect at a point known as the triple point (TP). At this point the three phases co-exist in equilibrium. In this region phase transitions take place when the temperature and/or pressure of the substance are changed. The vaporization curve starts at the triple point (TP) and ends at the critical point (CP) with co-ordinates, the critical pressure  $P_c$  and critical temperature  $T_c$ . The melting point curve starts at the triple point and rises steeply with increasing temperatures and pressures. Above the critical point the liquid and gas have the same density and no longer exist as separate phases. A further increase in pressure will result in an increase in density but no phase transition will take place. By increasing the temperature at constant pressure above the critical point there is a continuous transition from liquid to supercritical fluid or from gas to supercritical fluid by increasing the pressure of a gas at constant temperature. The region of pressures and temperatures above  $P_c$  and  $T_c$  in figure 4.1 is called the supercritical region and in this region a substance is said to be in a supercritical phase<sup>1</sup>. Table 4.2 lists the critical pressure and temperature for various solvents including the fluid density at the critical point known as the critical density ( $\rho_c$ ).



**Figure 4.1.** Typical (solid-liquid-gas -supercritical fluid) phase diagram

**Table 4.1** Comparison of the properties of supercritical CO<sub>2</sub> and those of gases and Liquids<sup>1</sup>.

	Density (g/cm <sup>3</sup> )	Viscosity (g/cm·s)	Diffusion coefficient (cm <sup>2</sup> /s)
Gases	0.0001-0.002	0.0001-0.0003	0.1-0.4
Supercritical CO <sub>2</sub> T <sub>c</sub> , P <sub>c</sub>	0.47	0.0003	0.0007
T <sub>c</sub> , 6P <sub>c</sub>	1.0	0.001	0.0002
Liquids	0.6-1.6	0.002-0.03	0.000002-0.00002

### 4.3 Supercritical fluid properties

Supercritical fluids exhibit physico-chemical properties intermediate between those of a liquid and a gas. SF properties include solvation, viscosity and diffusion coefficients. They are all influenced by density which is a function of applied pressure and temperature. These properties can be altered over a wide range by changing the pressure, the temperature or both simultaneously. A high density is responsible for increased solvating power of SFs, where interactions between the fluid and the solute molecules increase. At high densities, SFs have solvent strengths approaching those of liquids and they can dissolve many different types of solutes including thermally labile or high molecular mass and non-volatile compounds. Due to the lower densities of gases, they have no solvent action.

Supercritical fluids have more favourable hydrodynamic properties than those of liquids because supercritical fluid viscosity values are more like those of gases<sup>2</sup>. As a mobile phase in chromatography, gases have the fastest, liquids have the slowest and SFs have intermediate optimum flow rates. This is due to higher diffusion coefficients of analytes in gases as compared to supercritical fluids. Compared to LC, faster flow rates in SFC give rise to shorter analysis times. The diffusion coefficients of solutes in SFs are between those displayed for liquids and gases.

The solvent strength of supercritical fluids as a mobile phase in chromatography increases with compression. Densities may even approach those of liquids. The solvent strength depends on the average intermolecular distance, as defined by the density of the fluid. For liquid solvents, the density is generally constant with pressure and changes in the intermolecular distances of specific solvents can be considered negligible. The solubility power ( $\delta$ ), which was first introduced by Hildebrand and Scott<sup>3</sup> as a relative scale for solvent strength and a function of the cohesive energy density,  $c$ , is given as:

$$\delta = c^{\frac{1}{2}} = (\Delta u^{evp} / v)^{\frac{1}{2}} \quad [4-1]$$

Where  $\Delta u^{evp}$  is the evaporation energy and  $v$  is the molar volume. Giddings *et. al.*<sup>4</sup> extended this theory for its application in representing the solvating power of supercritical fluids as :

$$\delta = 1.25 P_c^{\frac{1}{2}} (P_r / P_{r,liq} ) \quad [4-2]$$

where  $P_c$  is the critical pressure,  $P_r$  the reduced density of the substance in the supercritical state and  $P_{r,liq}$  is the reduced density of the substance in the liquid state.

The density of the mobile phase is the most important parameter to influence and optimise for separations in SFC. Density programming during an analytical run is as common in SFC as temperature programming in GC or programming of eluent composition (gradient elution) in HPLC<sup>5</sup>. The influence of density on the solvent properties is demonstrated using the concept of the solubility parameter, first introduced by Hildebrand and Scott<sup>3</sup>. The solubility parameter values vary from 0 up to liquid-like values of 10 at high densities. To solubilize a solute compound, the solubility parameters of both the solute and solvent need to be of equal values. To use a SF as solvent, the pressure of the fluid must be higher than the critical pressure, at which the density becomes similar to that of the liquid<sup>2</sup>.

#### 4.4 Supercritical Fluid Chromatography

Supercritical fluid chromatography (SFC) is defined as a form of chromatography (i.e. a physical separation method based on partitioning of an analyte between the mobile phase and the stationary phase) in which the mobile phase is subjected to pressures and temperatures near to or above the critical point for the purpose of enhancing the mobile phase solvating power<sup>6</sup>. The use of SFs as a chromatographic mobile phase was first reported in 1962 by Klesper<sup>7</sup>.

Various fluids are used as supercritical mobile phases, for example, carbon dioxide, ammonia, sulfur dioxide, alcohols, chlorofluoromethanes and low boiling hydrocarbons. **Table 4.2** lists the critical pressure and temperature for various solvents including the fluid density at the critical point known as the critical density ( $p_c$ ). The solvent properties of SFs that are relevant to chromatography are the critical temperature, critical pressure and polarity. Any specific solute-solvent intermolecular interaction such as hydrogen bonding which can enhance solubility and selectivity in a separation can be used to alter selectivity.

Early developments in SFC were slow due to the experimental problems in using supercritical fluids, lack of commercially available SFC instrumentation and it being overshadowed by the simultaneous growth of LC<sup>8</sup>. The resurgence of interest in SFC was driven by the potential advantages afforded by the unique characteristics of the mobile phase in SFC over GC and HPLC, and more importantly it has been augmented by advanced technology in pumps and detectors for SFC.

The rapid mass transfer in a supercritical fluid mobile phase also attracted interest from researchers because it offers high speed separation capabilities. It has been used successfully with high resolution open-tubular capillary columns with internal diameters smaller than 100  $\mu\text{m}$  which was not so successful in liquid chromatography due to slow mass transfer and high back pressures. SFC has also been achieved successfully on packed capillary columns with internal diameters smaller than 1 mm<sup>9</sup>. One of the principal benefits of SFC is the flexibility of using both GC and HPLC detectors. For inorganic mobile phases such as carbon dioxide, ammonia and xenon, the universal flame ionization detector (FID) is commonly chosen. Modification of these supercritical fluids with more polar organic substances must be avoided since the modified phase will give a response in the FID. The absence of suitable pure supercritical fluids of high solvent strengths has probably been the main reason for a steady loss of interest in SFC.

**Table 4.2** Features of various solvents at critical temperature, pressure and density.<sup>10</sup>

Solvents	T <sub>c</sub> (°C)	P <sub>c</sub> (bar)	ρ <sub>c</sub> (g/mL)
<b>Inorganic</b>			
Carbon dioxide	31.1	72	0.47
Dinitrogen monoxide	36.5	70.6	0.45
Ammonia	132.5	109.8	0.23
Water	374.2	214.8	0.32
Sulfur hexafluoride	45.5	38	
Helium	-268	2.2	0.07
Xenon	17	56.9	1.11
<b>Hydrocarbons</b>			
Methane	-82	46	0.169
Propane	96.7	42.4	0.22
Ethylene	11	50.5	0.2
Benzene	288.9	98.7	0.302
Toluene	319	41.1	0.292
<b>Alcohols</b>			
Methanol	239	78.9	0.27
Isopropyl alcohol	235.3	47.6	0.273
<b>Ethers</b>			
Ethyl methyl ether	164.7	47.6	0.272
Tetrahydrofuran	267	50.5	0.32
<b>Halides</b>			
Trifluoromethane	26	46.9	0.52
Dichlorodifluoromethane	111.7	109.8	0.558
Chlorotrifluoromethane	28.8	214.8	0.58
Trichlorofluoromethane	196.6	28.9	0.554
<b>Miscellaneous</b>			
Acetonitrile	275	47	0.25
Pyridine	347	56.3	0.312



Compared to GC, capillary SFC can provide high resolution chromatography at much lower temperatures and allows fast analysis of thermally labile or high molecular mass compounds. Although GC and HPLC complement each other, they together are unable to cover all needs. A set of problems falls between the capabilities of GC and HPLC<sup>11</sup> :

1. *the analyte is either not volatile enough for GC or is thermally unstable and the analyte is present in a very complicated mixture (requiring a high efficiency separation) or*
2. *the analyte is either not volatile enough for GC or is thermally unstable and the analyte cannot be detected well enough in liquid mobile phase.*

In these cases, both HPLC and GC fail. The ideal technique to fill the gap should have a low temperature, solvating mobile phase of programmable strength, high chromatographic efficiency, and universal detection. SFC when used with CO<sub>2</sub> as mobile phase, is compatible with FID and has the characteristics needed to fill the gap with only one major restriction. The solute will have to have some minimum solubility in an FID compatible mobile phase<sup>12</sup>. Also the non-polarity of the CO<sub>2</sub> mobile phase poses some restrictions on analysing more polar compounds on packed silica gel columns<sup>13</sup>.

#### 4.4.1 Retention behaviour in SFC

The final resolution obtained in a chromatographic separation is a function of column efficiency, selectivity and retention as stated by the well known master resolution equation:

$$R_s = \frac{\sqrt{n}}{4} \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{k}{k + 1} \right) \quad [4-3]$$

where  $\alpha$ ,  $k$  are not constant along the column in SFC packed columns due to the pressure drop<sup>14</sup>.  $k$  is the retention factor and  $\alpha$  is the relative retention also known as the selectivity.

The understanding of the solute retention mechanism of SFC is dependent on determining the complex interaction between multiple chemical processes. These physico-chemical processes involve the temperature, pressure, density and intermolecular interactions of the solute molecule with the mobile phase and stationary phase.

The density of a supercritical fluid is the parameter that determines the solvation power of the mobile phase. If the density increases, then the solvent strength of the fluid is increased. Density programming during the analytical run is as common in SFC as temperature programming in GC or programming of the eluent composition (gradient elution) in HPLC. Pressure is also one of the fundamental properties that influences solvation strength of a fluid. Pressure is the physical property that is directly measured by supercritical fluid delivery systems. At a fixed temperature, when the pressure is increased, the density and solvent strength of the mobile phase increases. On the other hand, an increase in temperature at a fixed pressure causes the solvation strength of the fluid to decrease as the density decreases and this in turn increases the retention times. Temperature has an important influence on selectivity as far as group-type in SFC on silica gel is concerned. It has been shown that group-type separation strongly depends on temperature and that the best group separation is obtained at low temperatures<sup>15</sup>.

#### 4.4.2. Packed vs Capillary SFC columns

Both packed and capillary columns can be used in SFC to elute a wide range of compounds with some modifications on the system to suit conditions for a particular column. The total surface area of the packing in a packed column is much greater than the surface area of the capillary tube, giving it a larger sample capacity. Larger amounts of analytes can be separated and can be collected. Due to the shorter diffusion interparticle distances in packed columns, higher linear flow rates may be used.

Packed columns generate a greater number of theoretical plates per unit length, which together with the high linear flow rates permits faster analysis than in a 50-100  $\mu\text{m}$  i.d. capillary column. Due to smaller channel dimensions, decrease in the number of theoretical plates with an increase in flow rate is less for packed than for capillary columns<sup>16</sup>. For constant column dimensions, the pressure drop along a SFC column is typically ten times smaller than in liquid chromatography, however, ten times greater than in gas chromatography. The primary advantage of capillary columns in SFC is that they offer a greater number of theoretical plates than packed columns due to the long lengths that can be used with a given pressure drop. The low pressure drop and open-tubular nature of the capillary column allow very long columns to be employed, whereas only a limited length for packed columns can be used due to the high pressure drop occurring. However, Berger<sup>5</sup> demonstrated that a very high number of theoretical plates may be achieved by coupling eleven packed-columns in series and 220 000 theoretical plates were achieved with early-eluting peaks producing up to 298 plates/second.

There are two general types of partition methods in liquid chromatography unlike in SFC: reversed-phase and normal-phase. In reversed-phase LC, the stationary phase is non-polar (chemically modified silica) and the mobile phase is polar (water + organic modifier). This is an excellent set-up for solubilizing and separating polar solutes. In normal-phase LC, the stationary phase is polar (silica or chemically modified surface)

and the mobile phase is non-polar (hexane, ether). Nowadays packed columns (such as normal phase LC chiral columns) are widely applied in SFC for racemic mixture separation. Packed column SFC is considered to be a suitable replacement for normal-phase liquid chromatography, mostly for the separation of polar compounds. In normal-phase LC and in SFC silica gel and porous silica are often used. This silica gel is made by poly-condensation of silicic acid<sup>18</sup>. Further gelation and drying of silica gel leads to porous silica particles (microbead), available as spherosil, porasil or others. They often contain many surface silanols which are removed by heat treatment or deactivation agents such as inorganic salts<sup>19</sup>.

In reversed phase packed column SFC two types of sites contribute independently to retention with non-polar supercritical fluids such as carbon dioxide. These are the surface of chemically bonded packings which is always heterogenous, containing different concentrations of chemically bonded and free silanol groups<sup>20</sup>. It has been established that the interaction of sample proton donor / acceptor and dipolar functional groups with free silanol groups of the column packings causes the characteristic peak tailing and sample adsorption or degradation that occurs in packed column SFC with relatively non-polar fluids<sup>21,22</sup>.

#### **4.4.3 Group separation by PLOT column**

Essential oils contain oxygenates such as aldehydes, ketones, esters and alcohols. These compounds have large retention factors on silica gel and are therefore difficult to elute. However, with a backflush method<sup>13</sup> or the addition of the modifier to the pure supercritical CO<sub>2</sub> mobile phase<sup>21</sup> it is possible to elute them. Alternatively, it has been shown that the reduced phase ratio of a porous layer open-tubular (PLOT) column allows elution of oxygenates without modifier or back-flush methods<sup>22</sup>.

Because of the relatively strong interaction of the sorbents inside PLOT and packed columns with polar molecules of the analyte, the kinetics of adsorption and desorption

is slower for the polar than for the less polar analytes. Thus, the column efficiency for less polar molecules would be higher than for polar molecules<sup>23</sup>. Column selectivity is classified approximately by three types of interaction of sorbent with analyte: size, strong dipole (polarity) and polarizability. Interaction of analyte with porous silica PLOT columns involves polarizability selectivity. Therefore, the separation of polar molecules such as light alcohols, thiols, esters, ketones, ethers and aldehydes can be achieved<sup>24</sup>.

The capability of a PLOT column to separate the oxygenates has been demonstrated<sup>25</sup>. Figure 4.2 shows the difference between the packed and PLOT column in terms of the dimensions and phase ratio. The phase ratio,  $\beta$ , is defined as the amount of stationary phase relative to mobile phase.

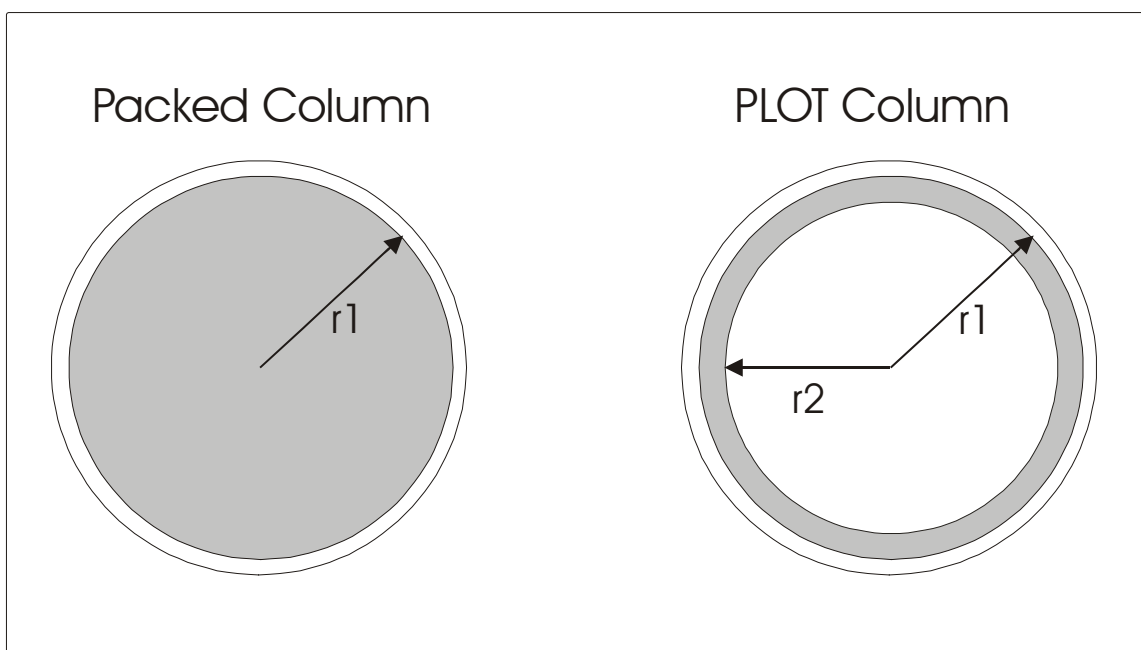


Figure 4.2 Schematic diagram showing the phase ratio difference in Packed and PLOT (specifically 0.3 mm id, 1.5  $\mu\text{m}$  porous layer) columns<sup>25</sup>.  $r_1$  (Packed) = 1 mm,  $r_1$  (PLOT) = 0.1500 mm and  $r_2$  (PLOT) = 0.1485 mm.

In PLOT columns most of the central volume available contributes to the volume filled with the mobile phase, this reduce the phase ratio. Since  $k = \beta K$ , retention factors are reduced 116 times<sup>25</sup>. The reduction in  $k$  makes it possible to elute polar oxygenated compounds with pure CO<sub>2</sub> on silica gel in PLOT columns.

#### 4.5. Conclusions

The advantages of supercritical fluids as mobile phase in chromatography have been shown in this chapter. These include the capability to work at room temperatures to express group-type separation of compounds at a given pressure range. It is important to note that separation temperature plays a role in order to achieve group separation. Group-type separation can be very important if applied to very complex mixtures, thus reducing sample complexity prior to second dimension analysis. With the opportunity to work with density programming, the mobile phase solvation strength can be manipulated. All these advantages of SFC will be applied in chapter 5 and 6 for group-type separation on essential oil samples.

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# CHAPTER 5

## SFC GROUP SEPARATION: EXPERIMENTAL

### 5.1 Introduction

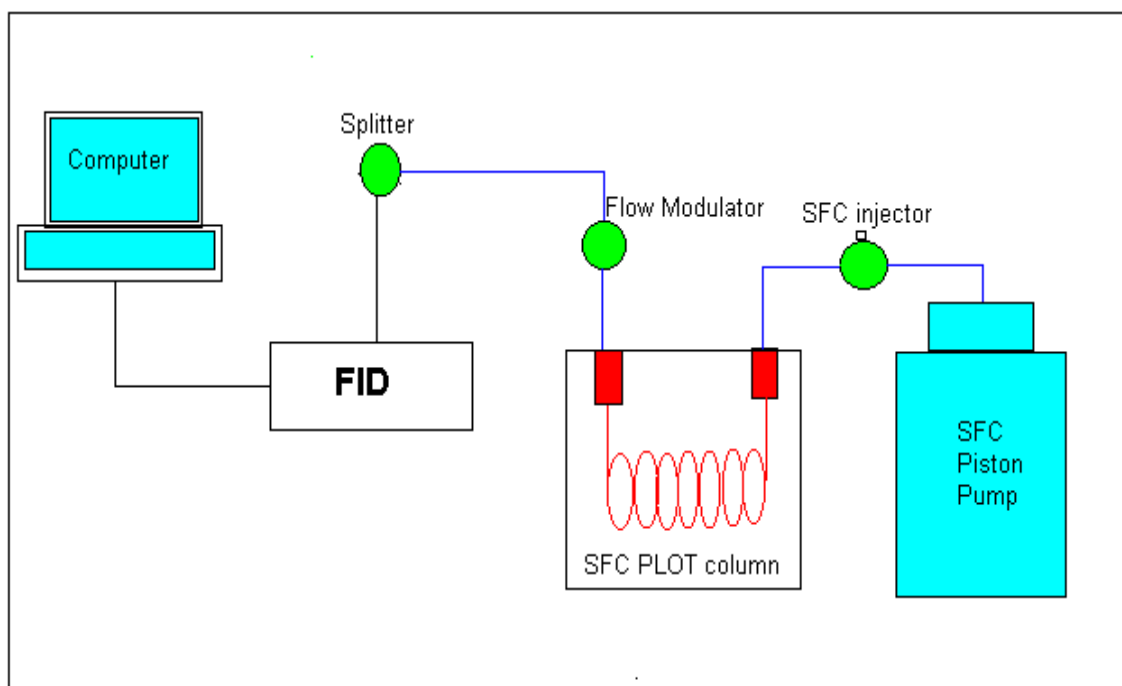
Supercritical fluid chromatography (SFC) can be defined as a form of chromatography (i.e. physical separation method based on partitioning of an analyte between the mobile phase and the stationary phase) in which the mobile phase is subjected to pressures and temperatures near or above the critical points for the purpose of exploiting the mobile phase solvation power<sup>1</sup>. Supercritical fluid chromatography can most easily be described as an adaption of either liquid chromatography (LC) or gas chromatography (GC) where the major modification is the replacement of either the liquid or gas mobile phase with a supercritical fluid mobile phase. SFC instruments employ almost all components normally used in conventional LC systems, including high-pressure pumps, stainless steel tubing, injection valves and columns with a few modifications or none at all<sup>2</sup>. By the 1980s dedicated SFC instruments were commercially available from a number of manufacturers. However, in recent years the use of SFC has declined.

In SFC the mobile phase is initially pumped as a liquid and is brought into the supercritical region by heating it above its critical temperature before it enters the analytical column. It passes through an injection valve where the sample is introduced into the supercritical stream, then into the analytical column. It is maintained at supercritical conditions as it passes through the column and into the detector by a pressure restrictor placed before a GC detector like the FID or after the LC type UV detector. The restrictor is a vital component as it keeps the mobile phase in the supercritical state throughout the separation. It often has to be heated at the exit to prevent clogging. Both variable and fixed restrictors are available.

In this chapter the application of SFC to separate compounds into different chemical classes using supercritical fluid CO<sub>2</sub> as the mobile phase is demonstrated with analysis of four different types of essential oils : *Tagetes minuta*, *Pelargonium*, *Artemisia afra* and *Cymbopogon*.

## 5.2 Instrumentation for SFC

The analytical system consists of a Lee Scientific (Salt Lake City, Utah, USA) Model 501 SFC pump to deliver supercritical fluid CO<sub>2</sub> (SFC grade, Air Products Sandown, South Africa) without helium head pressure to a Chrompack silica-gel PLOT column. An integral restrictor, prepared in the laboratory<sup>3</sup> was used at the column exit to the FID to maintain supercritical fluid pressure conditions. The isothermal column conditions were maintained by a PYE-Unicam GCD gas chromatograph. The FID was maintained at 280 °C. Chromperfect software (Justice Innovations California) was used for data acquisition. An actuated internal loop injector (Vici C14-W, Valco, Switerland) with an 0.2 µL internal loop was used for sample injection. All connections were made of 1/16" o.d. 120 µm i.d. stainless steel (SS) tubing with electropolished ends and connected with SS ferrules and connectors.



**Figure 5.1.** Schematic diagram of SFC instrument for group-type separation using a silica-gel PLOT column.

### 5.2.1 Restrictors for SFC

The use of either packed or capillary columns under SFC conditions requires a flow restrictor at the outlet of the column to maintain supercritical conditions of the mobile phase throughout the column. The ideal restrictor has to possess the following features<sup>4</sup>:

- 1. At the column or detector interface, the restrictor must effectively transfer the mobile phase and solute materials from a supercritical phase into a gaseous phase compatible with the detection mode.*
- 2. The desired restrictor should produce negligible extra-column zone broadening effects.*
- 3. Should also allow uniform, pulse free flow.*

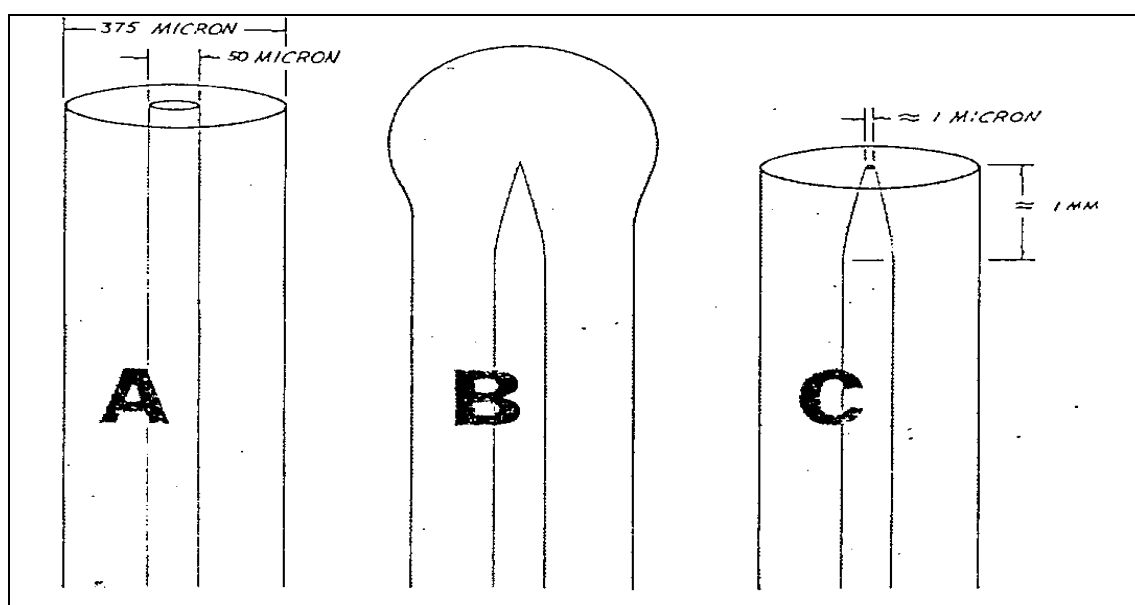
Other important practical aspects to be considered include the ease of restrictor fabrication and the restrictor mechanical durability. In SFC, the mobile phase pressure or density controls the solute partitioning phenomena while the restrictor geometry controls the mobile phase linear velocity<sup>4</sup>.

Increased restrictor temperature reduces the flow rate (mass flux) if the fluid is a gas. If the fluid is cooled to the liquid state in the restrictor, mass flux will increase with temperature. This behaviour is consistent with the temperature dependence on fluid viscosity. Studies have shown that at higher temperature (>120 °C) the linear velocity of supercritical CO<sub>2</sub> has nearly a direct dependence on pressure. If the SFC separation column and restrictor are independently thermostated, SFC mass flux (linear velocity times fluid density in the column) should be independent of the column temperature<sup>1</sup>.

The integral restrictors were fabricated according to the Guthrie and Schwartz process<sup>3</sup> depicted in figure 5.2. The capillary tube was heated so that the fused silica slowly drew closed by surface tension of the molten silica, producing a well defined conical closure using an oxygen-butane welding torch. The closed end of the capillary tube

was then gently abraded by hand using a wet abrasive sheet in a container of water, carefully removing the excess fused silica from the terminal end of the column until the conical closure was reached. The escape of gas bubbles from the pressurised column indicated when the conical closure point had been reached and the flow rate of the escaping gas was then measured. While polishing the capillary end, gas flow rate was measured until the desired flow rate was achieved. After the desired mobile phase flow rate was obtained the outlet of the restrictor column was inserted into the heated split/splitless injector of a GC (to prevent condensation and blockage of restrictor) and the other end into the FID for SFC analysis.

If excessive heat is used, then a rounded, rather than the proper conical, closure would be produced. The rounded closure is difficult to use, since when the polishing process reaches the fused silica inner wall, small polishing increments will produce rapid increases in the orifice diameter and flow rate.



**Figure 5.2.** Restrictor fabrication sequence process. (A) Capillary before heating. (B) Capillary after heating with microtorch. (C) Capillary terminus and orifice after wet polishing<sup>3</sup>.

### 5.2.2. Supercritical fluid CO<sub>2</sub> mobile phase

There are a number of possible fluids which may be used in SFC as the mobile phase. Pure carbon dioxide (CO<sub>2</sub>) is not polar, making it a mobile phase most appropriate for the elution of low to moderately polar solutes<sup>5</sup>. However, based on its low cost, low interference with chromatographic detectors, and good physical properties (non-toxic, non-flammable, low critical values), CO<sub>2</sub> is the standard mobile phase. The main disadvantage of CO<sub>2</sub> is its inability to elute very polar or ionic compounds. This can be overcome by adding a small portion of a second fluid called a modifier fluid. This is generally an organic fluid (alcohols, cyclic ethers). The addition of the modifier fluid improves the solvating ability of the supercritical fluid and sometimes enhances selectivity of the separation. It can also help improve separation efficiency by blocking some of the highly active sites on the stationary phase<sup>5</sup>. Modifier fluids are commonly used, especially in packed column SFC.

Sulphur hexafluoride (SF<sub>6</sub>) was investigated as an alternative to CO<sub>2</sub> as mobile phase for group separation<sup>6</sup>. SF<sub>6</sub> is a very weak solvent and less polarizable than CO<sub>2</sub>. Hydrogen fluoride (HF) produced when SF<sub>6</sub> decomposes in the flame is very corrosive to the FID. However, flame ionisation detection was made possible by gold plating of the detector. Xenon showed resolution of chemical groups comparable to that of CO<sub>2</sub>, with superior coupling to the fourier transform infra-red (FTIR) detector due to the absence of infra-red absorption bands from the mobile phase<sup>7</sup>. The fluid was however, found to be too expensive for routine analysis. Ammonia is suitable for more polar compounds, however, it is not compatible with the FID.



### 5.3 Optimization of SFC group separation

Optimization of the fluid for chromatographic separation can be obtained by changing a variety of parameters. In GC by changing or programming the analysis temperature and choosing a suitable stationary phase, the separation efficiency can be altered. On the other hand, in liquid chromatography, the variation and programming of the eluent composition or gradient elution are the vital tools for optimization beside the choice of the stationary phase, which is packed into a column as particles.

In SFC all parameters mentioned above for GC and LC have an influence on the separation efficiency. These are: (1) selection of a stationary phase ( in either a packed or capillary column), (2) selection of a mobile phase and (3) optimisation of the analysis temperature. Further, variation of the eluent density and the working pressure are of great importance in optimizing the SFC separation. Eluent composition, temperature, pressure (and therefore also density) may be varied individually or simultaneously. In the following paragraphs a brief and tentative optimisation study for group separation is reported. A detailed study would involve not one compound per class type but a volatility range of compounds for each class.

#### **5.3.1. Determination of the optimum flow rate**

Optimization of the separation conditions in chromatography requires careful evaluation of the influence of mobile phase flow rate on the peak resolution. The flow rate of the mobile phase is one of the most important factors to be taken into account in order to properly design and operate an SFC system.

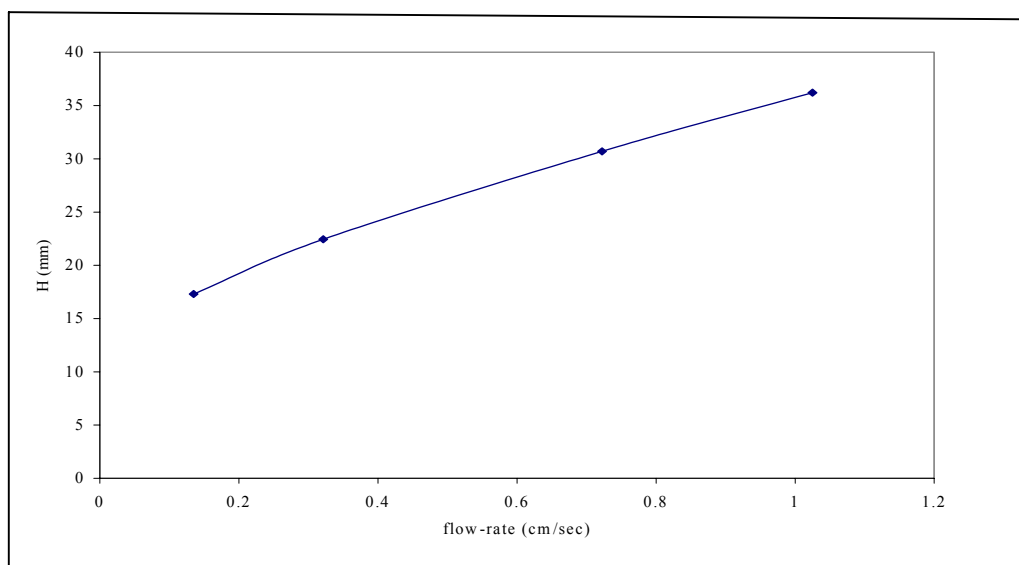
##### *5.3.1.1 Experimental*

Before starting with the investigation of the influence of pressure and temperature on the alkanes, ethers, esters, alcohols and aldehydes resolution, the PLOT column

optimum performance was tested. For this reason the Van Deemter curve was drawn up at a constant pressure of 150 atm and 28 °C near critical temperature with different sets of restrictors. Dodecanal in CS<sub>2</sub> solvent was injected using a different set of restrictors. The restrictor flow rate was changed at constant pressure of 150 atm and temperature 28°C.

### 5.3.1.2 Results and Discussion

Figure 5.3 shows the plate height, measured for the dodecanal peak versus volume flow rate. It can be seen HETP increases slightly with increasing flow. However, the optimum point of HETP could not be achieved despite using a very slow rate of 20 ml/min that ended in a long analysis time of about 9 hrs. This slow chromatography is expected due to the large inner diameter (0.32 µm) of the open tube by SFC standards, where 50-100 µm i.d's are the norm. To avoid the long analysis time resulting from the slow flow rate, the SFC separation was done at the flow rate of 480 ml/min (atmospheric pressure) measured at the restrictor exit. This corresponds to a linear flow rate of 7.7 cm/sec in the column.



**Figure 5.3.** Van Deemter curve for dodecanal flow rate vs Plate height at constant pressure of 150 atm and temperature 28 °C with different restrictor flow rates.

### 5.3.2 Effect of temperature on SFC group separation

The effect of temperature at constant pressure is even more complicated than the effect of pressure at constant temperature. Increasing temperature decreases fluid density and solute-fluid interaction, which results in a decreased solvation power. At the same time it also decreases solute-solute interaction, which results in an increased solubility. Therefore, in terms of temperature, two competing factors affect the capacity factor ( $k$ ) of solutes in the SFC separation<sup>1</sup>. In general there are two ways in which a change in temperature can influence resolution in SFC. Firstly, changing the temperature alters the density and consequently the solvent power of the fluid so that the capacity factors ( $k$ ) of solute changes, secondly, temperature can play a deciding role in selectivity ( $\alpha$ ).

It has been found that a small change in temperature can result in large changes in resolution and retention<sup>8,9</sup>. Further, the selectivity ( $\alpha$ ) changes with temperature in open-tubular SFC<sup>5</sup>. This behaviour is important because it provides the means to adjust the selectivity over a fairly wide range. The suitable temperatures for the PLOT column separation were investigated in relation to the elution and resolution of groups in essential oils samples. It is important that a suitable temperature is determined where only polarity and not volatility is expressed.

#### 5.3.2.1. Experimental

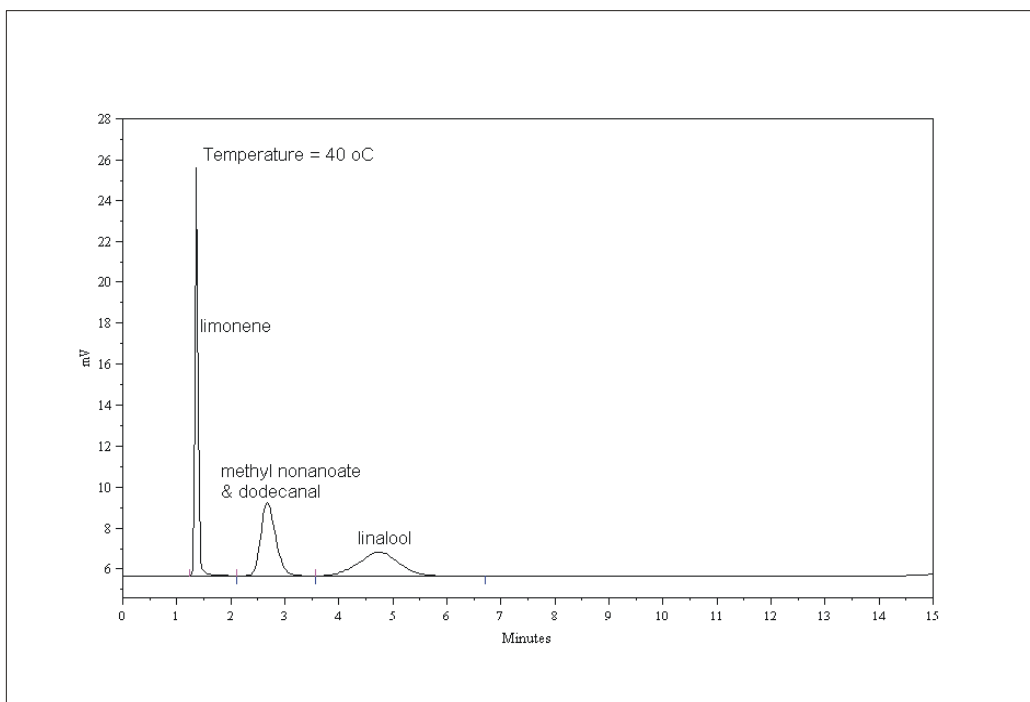
A 0.2  $\mu$ l standard mixture containing limonene, methyl nonanoate, iso-amyl ether, dodecanal and linalool was prepared in 500  $\mu$ l CS<sub>2</sub> and injected at a constant pressure of 110 atm and temperatures (20 °C, 28 °C and 40 °C). To reduce long analysis times, a high flow rate of 480 ml/min (atm) measured at the column exit was used.

### 5.3.2.2 Results and Discussion

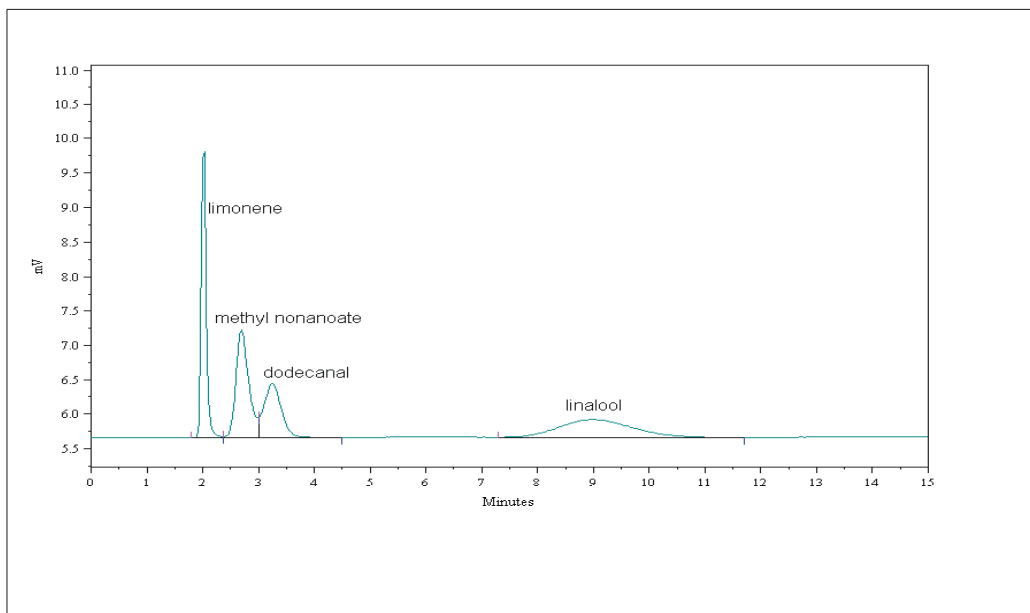
Figures 5.4 to 5.6 show the SFC chromatograms of the standard sample at different temperatures (20 °C, 28 °C and 40 °C). The pressure of 110 atm was used to investigate the effect of temperature on group separation. These chromatograms were obtained in about 20 minutes run-time. This was achieved by very fast linear flow rates measured as 480 ml/min decompressed CO<sub>2</sub> at the column exit which correspond to a linear flow rate of 7.7 cm/sec. It has been documented that operation below the critical temperature is beneficial for group-type separation in SFC<sup>10,11</sup>. At a temperature 40 °C the ester and the aldehyde co-elute as shown in figure 5.4. The trends of increased group selectivity at the lower temperature (30 °C) is followed as predicted by the literature<sup>10,11</sup>. However, it appears from the observations that group selectivity of esters and aldehydes is more efficient at the temperature of 28 °C compared to both 20 °C and 40 °C. Table 5.1 shows the calculated capacity factor [ $k=(t_r - t_m)/t_m$ ] values as the function of temperature of the methyl nonanoate and dodecanal by taking limonene as the unretained molecule.

**Table 5.1.** Change in capacity factor of esters and aldehydes groups as a function of temperature.

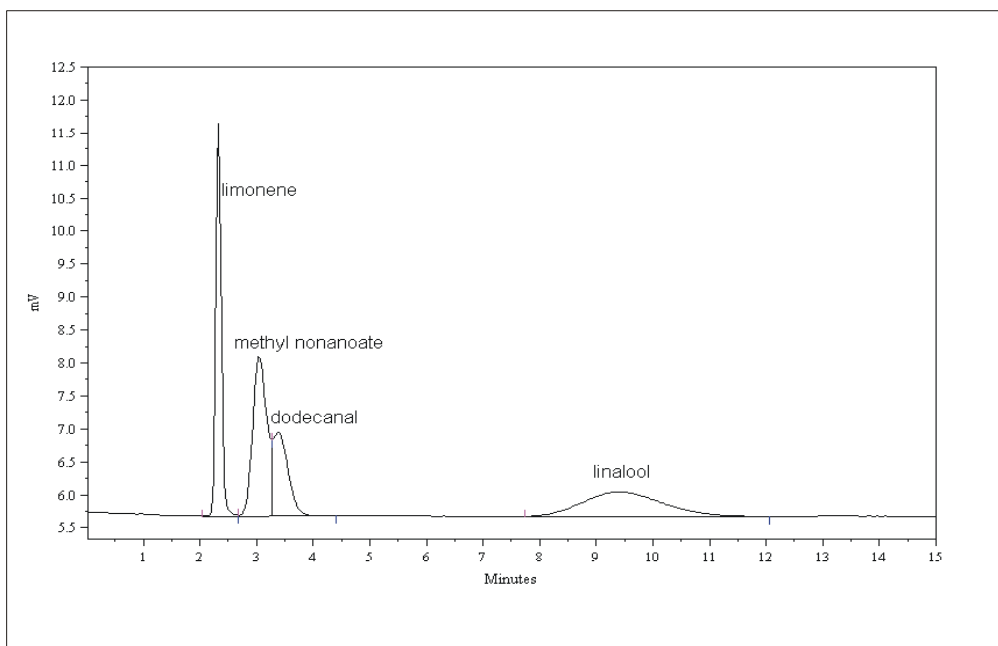
Compounds	20 °C	28 °C	40 °C
Limonene $t_m$	0	0	0
Methyl nonanoate	0.31	0.33	0.27
Dodecanal	0.45	0.61	0.39



**Figure 5.4.** SFC<sub>PLOT</sub> group separation. Pressure 110 atm and temperature 40 °C.



**Figure 5.5.** SFC<sub>PLOT</sub> group separation. Pressure 110 atm and temperature 28 °C.



**Figure 5.6.** SFC<sub>PLOT</sub> group separation. Pressure 110 atm and temperature 20°C.

### 5.3.3 Effect of pressure on SFC group separation

While density is the fundamental property that influences solvation strength of a supercritical fluid, pressure is the physical property that is directly measured by supercritical fluid delivery systems. In SFC retention of the solute is closely related to the increase in the solvent strength of the mobile phase during the run. At fixed temperature, when the pressure is increased, the solvent strength of the mobile phase increases as the density increases.

#### 5.3.3.1 Experimental

A standard sample mixture containing compounds of different chemical classes was prepared into 500  $\mu\text{l}$   $\text{CS}_2$  solvent and injected at different pressures and a constant temperature of 28 °C to investigate the effect of the mobile phase pressure on group-type separation by SFC<sub>PLOT</sub> column. Table 5.2 shows the standard compounds prepared.

**Table 5.2.** Different chemical compound class standard prepared for studying the pressure effect on SFC<sub>PLOT</sub> group-type separation.

<b>Hydrocarbon</b>	<b>Ester</b>	<b>Ether</b>	<b>Aldehyde</b>	<b>Alcohol</b>
Limonene	Methyl nonanoate	Iso- amyl ether	Dodecanal	Linalool

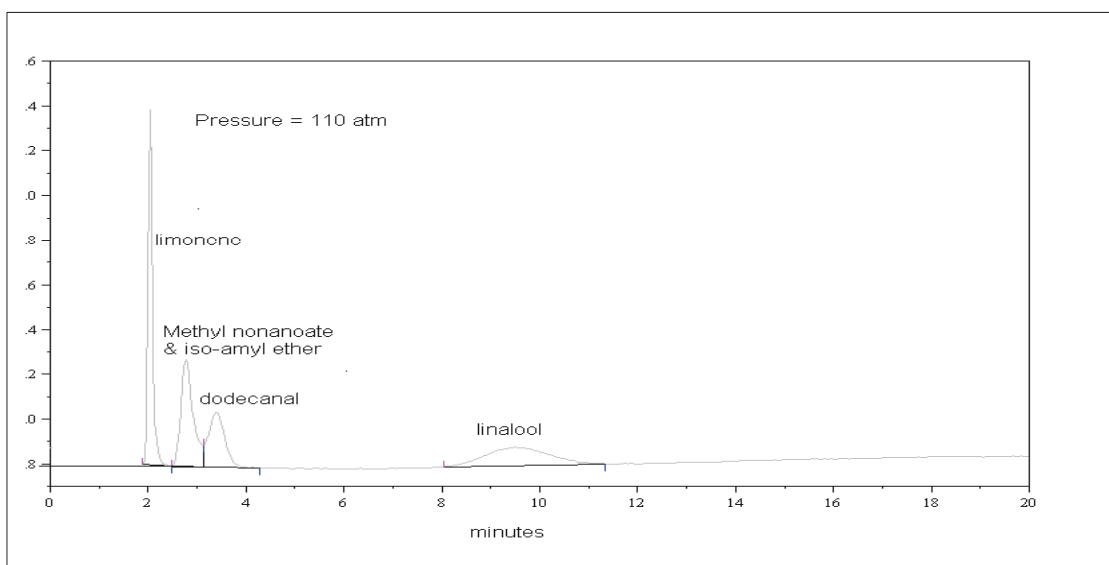
### 5.3.3.2 Results and Discussions

Figure 5.7 to 5.11 shows the SFC group separation chromatograms of the standard sample mixture of different compounds. Under the chromatographic conditions mentioned above, limonene (terpene hydrocarbon) was eluted in the first fraction because of less molecular interaction with the silica gel PLOT column surface. Methyl nonanoate (ester) and iso-amyl ether (ether) co-elute in the second fraction while the aldehyde compound dodecanal eluted in the third fraction. An alcohol, linalool eluted last due to the strong retention on the silica-gel surface.

With a silica-gel PLOT column, hydrocarbons are well separated from the carbonyl and oxygenate compounds because of the differences in polarity of the stationary phase and the mobile phase. At lower pressures, below 120 atm, methyl nonanoate and iso-amyl ether are better separated from dodecanal. Compounds are ordered according to the functional group i.e. group selectivity is strongly expressed. The situation becomes slightly worse as the pressure increases. The resolution of methyl nonanoate and iso-amyl ether with dodecanal is slightly reduced.

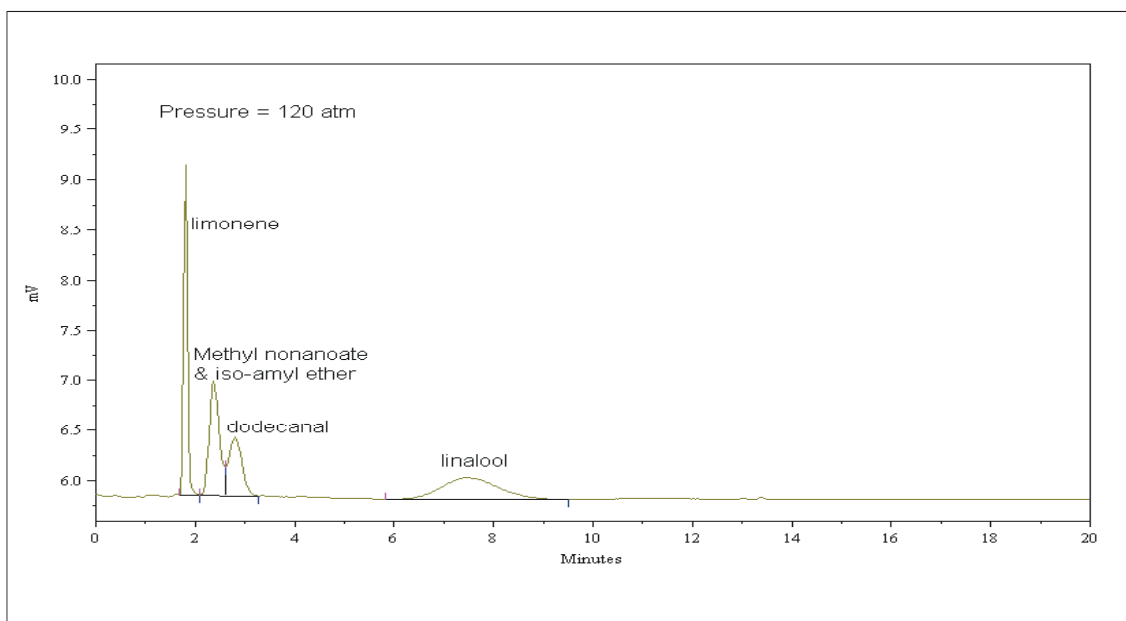
It appears from the observations in figure 5.7 to 5.11 that analytes elute earlier as the solvent strength of CO<sub>2</sub> increases with increased pressure as expected (also because of a slight increase in mobile phase linear flow rate). In this case, the use of density programming of the SFC mobile phase like temperature programming in GC and gradient elution in LC will solve the problem. The group selectivity between ester and aldehydes seems better at lower pressure, so it could be better to start the separation at

lower pressures and ramp to higher pressure gradually. By increasing the pressure using the pressure or density programming, both flow rate and the solvent strength will be enhanced to elute certain compounds. Thus, polar compounds that are strongly retained on the phase can be chromatographed in relatively short time without the loss of resolution for the earlier eluting compounds.

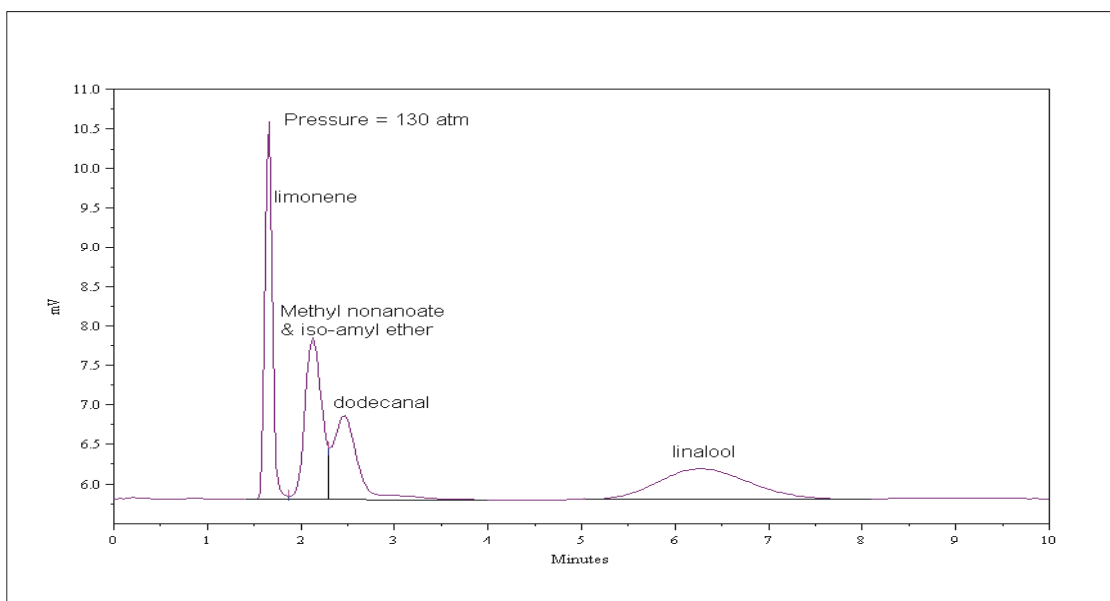


**Figure 5.7.** SFC<sub>PLOT</sub> chromatogram of standard sample at pressure 110 atm.

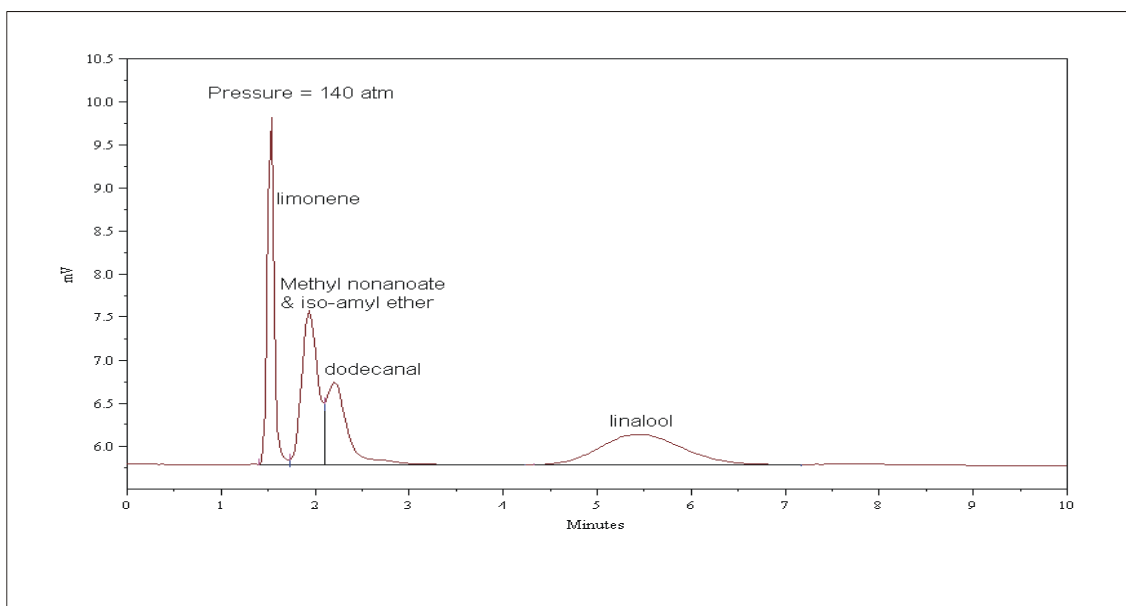




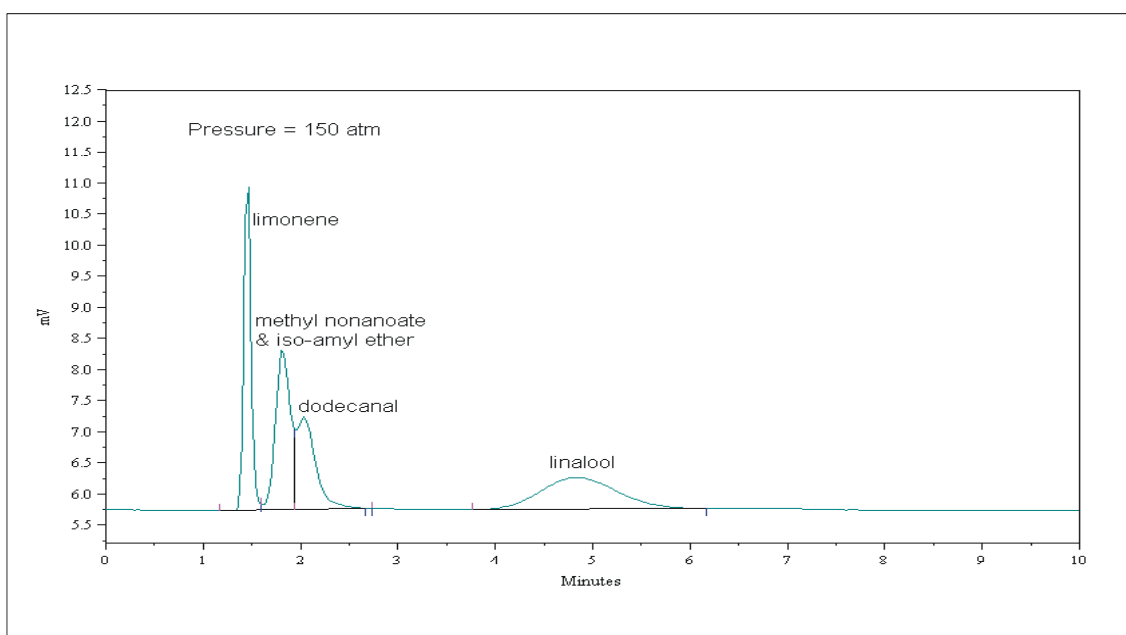
**Figure 5.8.** SFC<sub>PLOT</sub> of standard sample at pressure 120 atm.



**Figure 5.9.** SFC<sub>PLOT</sub> of standard sample at pressure 130 atm.



**Figure 5.10.** SFC<sub>PLOT</sub> of standard sample at pressure 140 atm.



**Figure 5.11.** SFC<sub>PLOT</sub> of standard sample at pressure 150 atm.

### 5.3.4 Analysis of essential oil sample

#### 5.3.4.1 Experimental

Five pure samples of essential oils (*Tagetes minuta*, *Artemisia afra*, *Pelargonium raden X capitatum*, *Cymbopogon citratus*, and *C. flexuosus*) were obtained from the Lowveld College of Agriculture. A 0.2 µl of real sample was injected undiluted into the optimized SFC for group separation. SFC conditions for group separation of the oil samples were used as outlined below.

**Table 5.3.** SFC<sub>PLOT</sub> optimized chromatographic conditions for group separation of essential oil samples

Temperature	28 °C
Pressure	110 atm
Flow rate	480 ml/min (measured at restrictor outlet)

#### 5.3.4.2 Results and Discussions

Four essential oils samples : *Cymbopogon citratus* and *C. flexuosus*, *Tagetes minuta*, *Artemisia afra* and *P. radens X capitatum* were successfully separated into different chemical classes of the terpene hydrocarbons and the oxygenates on a silica-gel PLOT column. The oxygenate derivatives were further separated into aldehydes, ketones, esters and alcohols using the CO<sub>2</sub> mobile phase. Figure 5.12 to 5.15 shows the SFC<sub>PLOT</sub> column chromatograms of the four different oils. The isobaric pressure of 110 atm and constant temperature of 28 °C conditions indicated in table 5.3 were used for this analysis. A common observation about all four oils is that they all contain terpene hydrocarbons, esters, ketones and aldehydes.

The absence of the alcohol chemical class in *T. minuta* and *A. afra* oils distinguish them from the other two oils (*Pelargonium* and *Cymbopogon*). This information could easily be obtained by SFC with a silica-gel PLOT column. The separation of compounds into different chemical classes is its main advantage compared to other separation techniques. Under the separation conditions in table 5.3 the elution order of the chemical classes is based on the polarities of the solutes and is similar to that in normal-phase liquid chromatography.

The compounds which belong to the same chemical class should ideally produce a single peak on the SFC with FID quantitation. Fraction I belongs to the terpene hydrocarbons group, the least retained on silica-gel PLOT column, followed by the carbonyl compounds. Information such as the quantitative amount of each group in the oil can readily be obtained in SFC. Note that in fig 5.14 and table 5.4 lemongrass shows a higher percentage of alcohols than other oils. This cannot be as this oil mainly consists of the aldehydes neral and geranial: more about this contradiction in chapter 6. This is consistent with the SFC data shown by Yamauchi where neral and geranial elute slightly before terpineol from the short (5 cm) packed silica gel column with CO<sub>2</sub> as mobile phase.

**Table 5.4.** Qualitative chemical group analysis in *Artemisia afra*, *Tagetes minuta*, *Pelargonium* and *Cymbopogon* oils. ( SFC Temperature = 28 °C and Pressure =110 atm)

<b>Fraction</b>	<b>RT(min)</b>	<b>Chemical Class</b>	<b>Artemisia</b>	<b>Tagetes</b>	<b>Pelargonium</b>	<b>Cymbopogon</b>
1	0-2.5	Terpenes	7.08	61.17	28.87	5.29
2	2.5– 12.0	Esters	16.87	5.99	18.20	0.40
		Ketones	61.170	5.48	8.90	4.57
		Aldehydes	14.88	27.42	12.09	2.75
3	>12.0	Alcohols			31.94	86.99

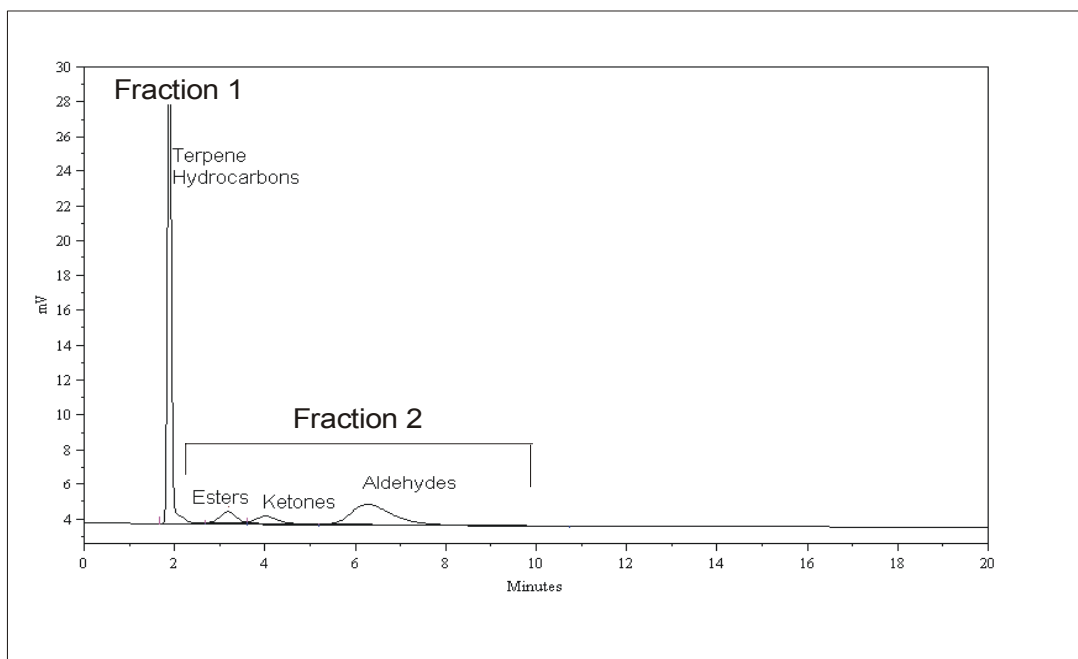


Figure 5.12. SFC<sub>PLOT</sub> chemical class separation of *Tagetes minuta* oil.

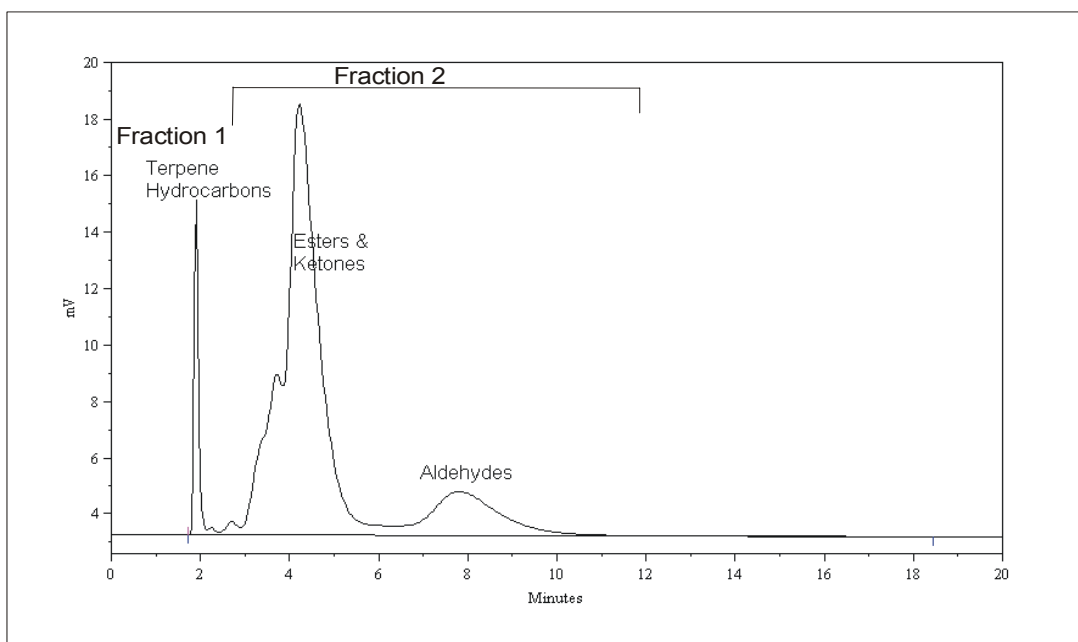
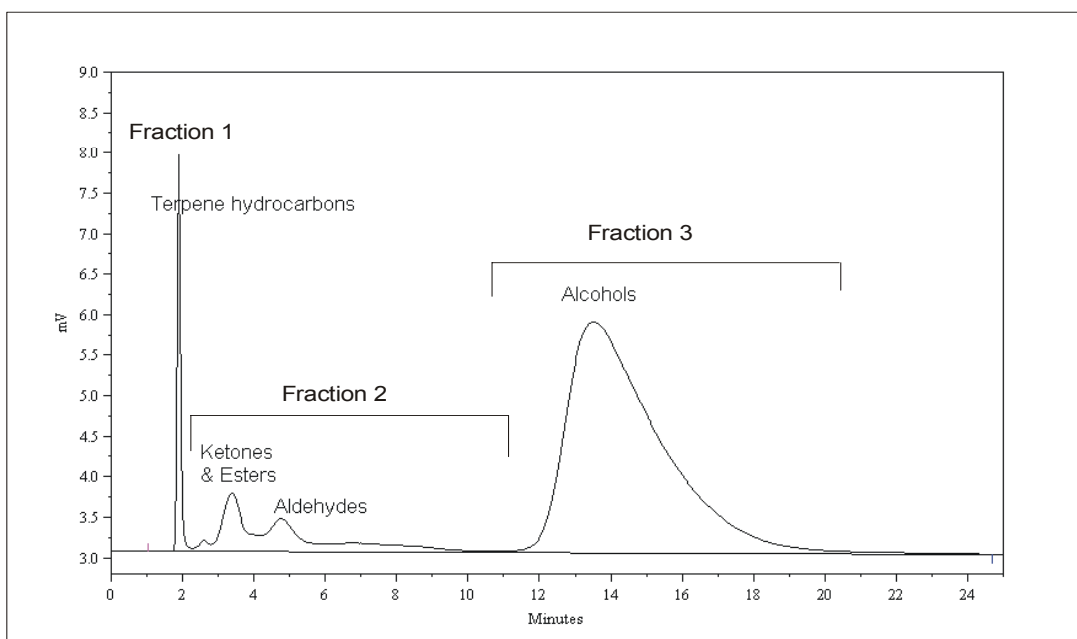
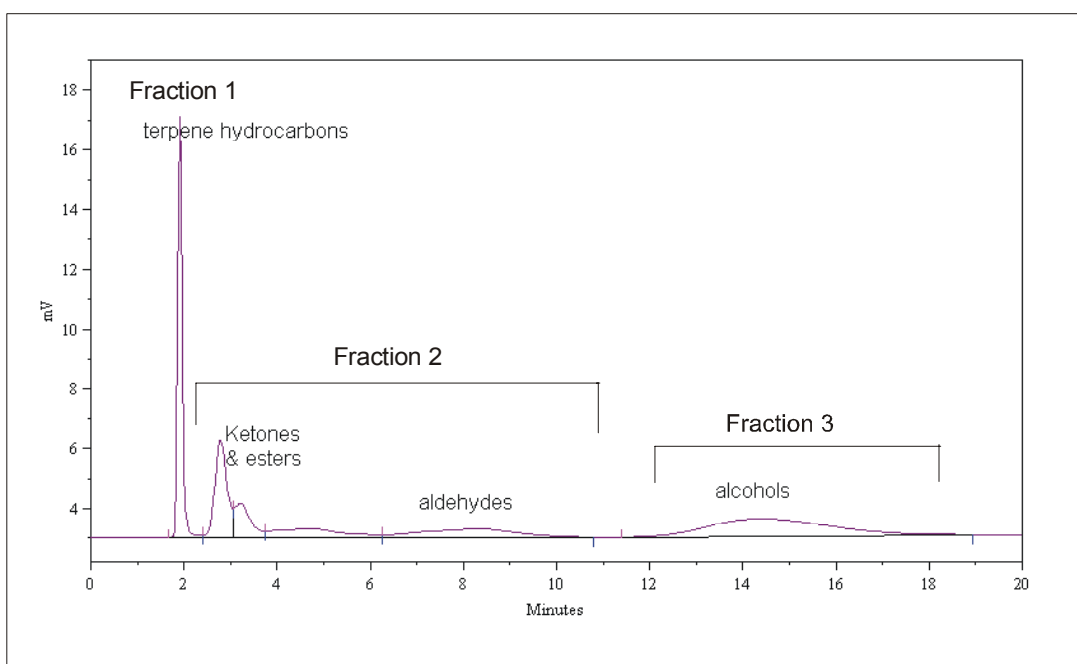


Figure 5.13. SFC<sub>PLOT</sub> chemical class separation of *Artemisia afra* oil.



**Figure 5.14.** SFC<sub>PLOT</sub> chemical class separation of *Cymbopogon* essential oil



**Figure 5.15.** SFC<sub>PLOT</sub> chemical class separation of *Pelargonium radens X capitatum* essential oil .

## 5.4 Conclusion

The results presented in this chapter shows that supercritical fluid chromatography with non-polar CO<sub>2</sub> mobile phase on a silica gel PLOT column is an appropriate technique for group-type separation. By using a silica-gel PLOT column, essential oil samples of *Cympogon flexuosus* and *C. citratus*, *Tagetes minuta*, *Artemisia afra*, and *P. radens X capitatum* are separated into terpenic hydrocarbons and oxygenate groups. The oxygenates are further separated into esters, aldehydes, ketones, aldehydes and alcohols. Group-type separation on a silica-gel PLOT column with SFC is obtained without the use of (1) the modifier or (2) backflush method.

For better group separation, it is important that the flow rate, separation pressure and temperature are investigated or optimized. Slightly, better group separation was found at a pressure of 110 atm and the near critical temperature of 28 °C. A flow rate of 7.7 cm/s [480 ml/min (atm) after expansion] was used to provide acceptable analysis times. The Van Deemter curve showed that higher chromatographic performance can still be expected at lower flow rates from the 0.32 i.d. PLOT column ( optimum SFC is normally performed in 100 µm i.d. columns or smaller). Although a thorough and systematic optimization was not our aim, convenient separation conditions could be found to couple the PLOT silica gel SFC group separation system to a second dimension GC as reported in the next chapter.



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# CHAPTER 6

## SFCXGC: EXPERIMENTAL

### 6.1 Introduction

Complex samples require analytical methods of high resolving power to provide reliable analysis of the sample components. This cannot be achieved in a single separation step. Sample pre-separation or clean-up is required for more complex mixtures and the use of successive chromatographic methods may be necessary. The advantages of multidimensional coupled chromatographic techniques have been demonstrated in a variety of studies<sup>1,2</sup>. It is now not uncommon to find comprehensive multidimensional techniques such as LCxLC<sup>3</sup>, GCxGC<sup>4</sup>, SFCxGC<sup>5</sup> and fast GC time-of-flight mass spectrometry (GC-TOF-MS)<sup>6</sup>.

Essential oils are too complex for direct analysis on a single separation method such as capillary GC. This chapter aims to show that SFC and resistively heated GC can provide comprehensive two-dimensional analysis when they are coupled together. As shown in the previous chapter, group separation of essential oil samples by SFC-FID already provides useful information on the relative percentages of terpene hydrocarbons, esters, ketones, aldehydes and alcohols. To increase the amount of data that can be obtained from essential oils, additional analysis of the SFC groups is necessary.

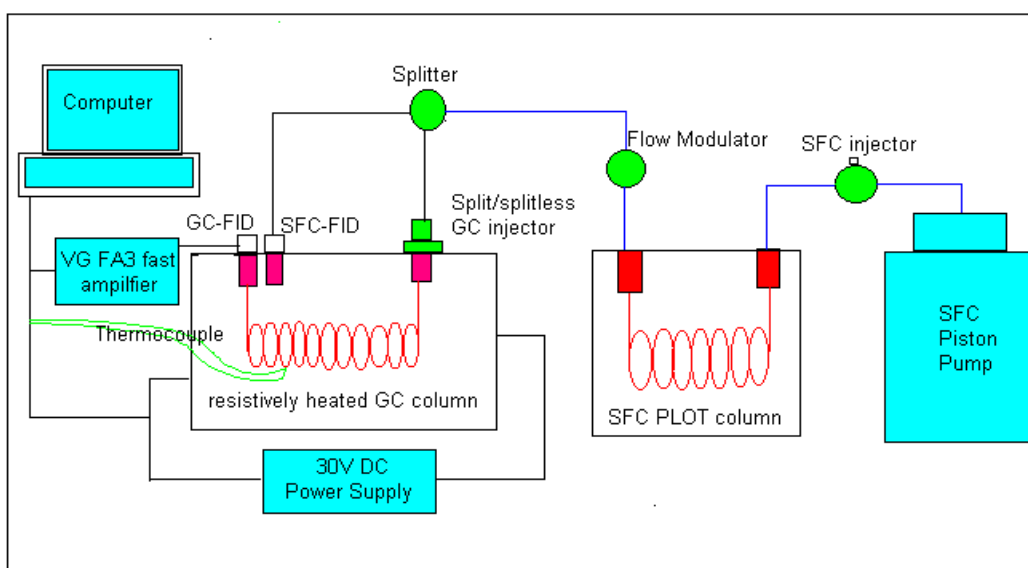
## 6.2 Instrumentation for SFCxGC

The analytical system<sup>7</sup> consist of a Lee Scientific (Salt Lake City, Utah, USA) model 501 SFC pump to deliver supercritical fluid CO<sub>2</sub> without He head pressure (SFC grade, Air Products, Sandown, South Africa) to a Chrompack silica-gel porous layer open-tubular (PLOT) column. The SFC is coupled to a Varian 3300 gas chromatograph (Varian Instrument Corporation), modified for resistive heating of a 1 m stainless steel capillary column, equipped with two FID's and a flow modulator. The SFC column exit was connected to a six-port valve (Vici, CW 6-K, Valco) flow modulator to cut small consecutive sections from the first dimension separation for transfer to the second dimension.

Fixed restrictors prepared in the laboratory<sup>8</sup> were used to maintain supercritical fluid pressure conditions throughout the column and the six port valve. Two integral restrictors were connected to the outlet of the flow modulator by means of a tee-junction splitter (Valco PN: ZT1C, Valco, Switzerland), one for SFC separated group quantitation directly by FID and the other to allow on-line collection of separated groups into the fast GC split/splitless injector by means of pressure drop focussing<sup>7</sup>. Pressure drop focussing occurs when the CO<sub>2</sub> density drops at the restrictor to focus the sample fraction on the head of the second column due to loss of solvation power of the CO<sub>2</sub> mobile phase.

A Pye-Unicam GCD gas chromatography oven maintained the isothermal SFC column temperature condition. On the Varian 3300, both FIDs for SFC and fast GC analysis were kept at 280 °C and the GC injector was kept at 250 °C. The resistively heated column was ramped from -50 °C to 300 °C at the rate of 450 °C/min (7.5 °C.s<sup>-1</sup>). Liquid CO<sub>2</sub> (Afrox, JHB, South Africa) was used to cool the Varian GC oven temperature down to -50 °C. A tightly coiled (1 m x 0.25 mm x 0.25 µm) SE-30 stainless-steel column (Quadrex Corporation SS Ultra Alloy) was used for GC analysis. A 30 Volt power supply was used to resistively heat the GC column<sup>7</sup>.

A thermocouple constructed from type K thermocouple wire having a diameter of 0.025 mm (25  $\mu\text{m}$ ) (Goodfellow, Cambridge GB) was glued to the exterior of the column with a drop of polyimide resin (Aldrich) to measure the column temperature. The temperature was controlled by Proportional Integral Derivative (PID) feedback on the thermocouple signal using a program written in LabView (version 5.1.1) Software<sup>7</sup>.



**Figure 6.1.** A schematic diagram of SFCxGC instrument.

### 6.2.1 Data Acquisition and Interpretation

All data were acquired using Chromperfect (version 3.7.4.0) Software package (Justice Innovations, California, USA). Short sections of 5 s duration were repeatedly collected from the SFC for the entire duration of the SFC run. Peak widths in the second dimension (resistively heated GC) are typically 0.5 s. Data collection rate (A/D converter) was at frequency of 100 Hz from a fast acting electrometer obtained from an old VG mass spectrometer<sup>7</sup>. Each GC run was recorded as a separate chromatogram. After the SFCxGC run was completed, data from the different chromatograms were compiled into a single text matrix file by a program written with LabView software (National Instruments, Texas, USA). Each 5 second cut from SFC (sum of peaks of one fast GC) constituted a data point that could be used to reconstruct the SFC

chromatogram. For visualisation, the matrix file was then imported into Transform 2D (Version 3.4, Fortner Research LLC, Sterling, VA, USA) software. Chromatograms were plotted by the use of Transform (Research Systems, Noeys Version V2.0) and MATLAB Version 6.0.0.88 (Mathworks Inc., USA) software packages<sup>7</sup>.

## 6.3 Experimental

### 6.3.1 SFCxGC analysis of essential oil standard

#### 6.3.1.1 Method and Conditions

A standard mixture containing 0.2  $\mu\text{L}$  each of selected essential oil components was prepared in 500  $\mu\text{L}$   $\text{CS}_2$  solvent and injected into an optimized SFCxGC for analysis.

**Table 6.1.** Standard prepared for essential oils analysis.

Terpene Hydrocarbons	Esters	Ketones	Aldehydes	Alcohols
<b>Monoterpenes</b>				
$\alpha$ -Pinene	Linalyl acetate	Methone	Citral	Geraniol
p-Cymene		Carvone	Citronellal	Linalool
Phellandrene		Camphor		4-terpineol
Limonene				
<b>Sesquiterpenes</b>				
Chamazuelene				

### Chromatographic method and conditions

#### SFC conditions

Column temperature 28°C, column flow rate 480 ml/min (7.7 cm/sec), pump pressure programming (110-200 atm at 1 atm/min)

#### Fast GC

Column temperature ramp (-50 °C to 300 °C) at 450°C/min, H<sub>2</sub> carrier gas flow rate 100 cm.s<sup>-1</sup>.

### **Modulation**

Cooling of the oven to  $-50\text{ }^{\circ}\text{C}$ , SFC fraction collection time 5 seconds, equilibration time 5 seconds, and followed by 50 s SFC stop flow condition (during which the GC is recorded).

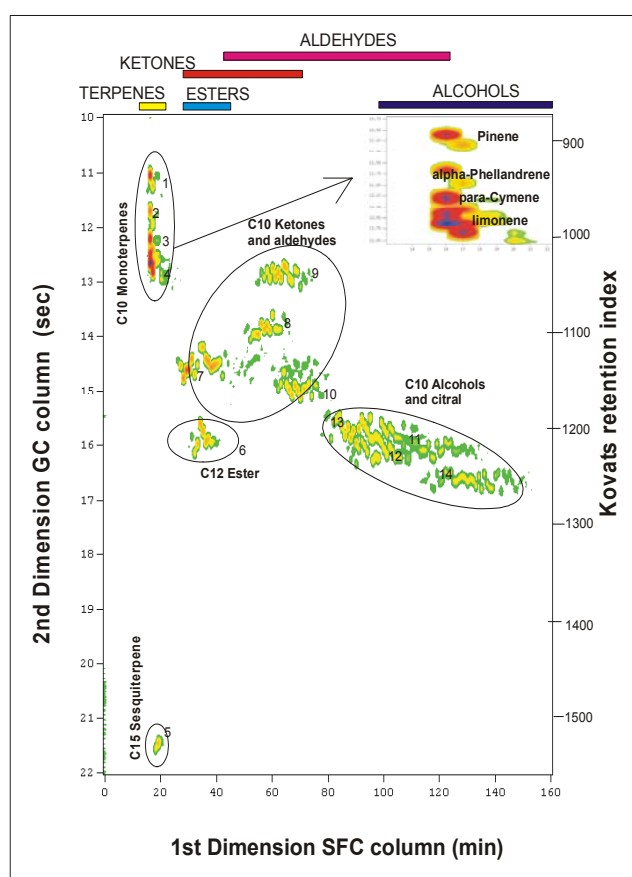
#### *6.3.1.2 Results and Discussion*

Figure 6.2 shows a typical polarity vs volatility SFCxGC chromatogram of the essential oil standard. On a two-dimensional plane, each compound forms a contour peak defined by the retention times of the two independent columns. The horizontal axis represents the retention on the polarity separation (SFC) and the vertical axis represents retention on the volatility analysis (GC). The chemical class separation of the compounds is obtained with the non-polar compounds (terpene hydrocarbons) eluting first on the first dimension axis due to their weak retention on the silica gel PLOT column phase, followed by the more polar oxygenated compounds. The oxygenated compounds are further separated into co-eluting esters and ketones followed by co-eluting aldehydes and alcohols. Terpene hydrocarbon compounds that elute together in the SFC dimension are separated into individual compounds in the second dimension based on their volatility. This group is thus separated into the closely spaced monoterpenes (C10 hydrocarbons), and the sesquiterpene (C15 hydrocarbons) eluting much later.

The first dimension axis, the SFC chromatogram, was developed in the conventional manner with pressure programming of the  $\text{CO}_2$  mobile phase from 110 atm to 200 atm at 1 atm/min and a constant temperature of  $28\text{ }^{\circ}\text{C}$ . About 160 secondary chromatograms were generated continuously at fixed 5 seconds collection time intervals by flow modulator from the first column.

Since the separation mechanisms as well as the physical state of the mobile phases are different in each dimension, compounds that are not separated on the first column are

likely to be separated on the second column. The width of the peaks associated with the later eluting 1<sup>st</sup> dimension compounds 11-14 can be compared to the SFC chromatogram in figure 5.14. The “patched” appearance of peaks from single compounds should not be confused with multiple peaks and result from imperfect reproducibility of the fast, temperature programmed GC runs. The most important feature in the chromatogram of figure 6.2 is the orthogonal separation achieved by using the two different separation mechanisms. Table 6.2 shows the names of the essential oil standard components.



**Figure 6.2.** SFCxGC chromatograms of essential oil standard analysis. SFC<sub>PLOT</sub> pressure ramp 110-200 atm at 1 atm/min, temperature =28 °C, Modulation: 5 s collection time and equilibration time = 5 s, Fast GC ramped -50 to 300 °C at 450 °C/min. Scale for Kovats index (1.19 sec = 100 units) calculated from peak 1 (Pinene, KI = 942) and peak 14 (Geraniol, KI =1234)

**Table 6.2.** Composition, chemical class, molecular weight, and retention data<sup>14</sup> for essential oil standard (Peak numbers refer to identified peaks in figure 6.2).

*I*=Kovats index on methyl silicone, *T*= isothermal temperature at which the index was determined, or "prog" if the index was determined using temperature programming<sup>14</sup>

Peak No.	Compound	Chemical class	MW	I <sup>14</sup>	T <sup>14</sup>
1	$\alpha$ -Pinene	C10 Monoterpene	136	942	100
2	$\tilde{\alpha}$ Phellandrene	C10 Monoterpene	136	1007	110
3	p-Cymene	C10 Monoterpene	136	1016	100
4	Limonene	C10 Monoterpene	136	1025	100
5	Chamazuelene	C15 Sesquiterpene	186		
6	Linalyl acetate	C12 Ester	196	1240	130
7	Menthone	C10 Ketone	154	1158	130
8	Camphor	C10 Ketone	154	1126	110
9	Citronellal	C10 Aldehyde	154	1017	Prog
10	Carvone	C10 Ketone	154	1228	Prog
11	Linalool	C10 Alcohol	154	1097	135
12	Terpineol	C10 Alcohol	154	1129	135
13	Citral	C10 Aldehyde	152		
14	Geraniol	C10 Alcohol	154	1234	175

**Reference:**<sup>14</sup> N.W. Davies, *J. Chromatogr.*, 503(1990) 1-24, for Kovats retention indices.



### 6.3.2 SFCxGC analysis of essential oil samples

#### 6.3.2.1 Method and conditions

Real essential oil samples of *Cymbopogon citratus* and *C. flexuosus*, *Artemisia afra*, *Pelargonium radens X capitatum*, and *Tagetes minuta* oil were obtained from Dept. of Agricultural Conservation and Environment (Lowveld Agricultural College, Nelspruit) sourced from different farms. A 0.2  $\mu\text{L}$  of each essential oil sample was injected, undiluted into an optimized SFCxGC system for group-type and individual compound separations. The same chromatographic conditions outlined in section 6.3.1.1 were used and the qualitative results of different oils were compared.

#### 6.3.2.2 Results and conditions

Lists of possible compounds occurring in the essential oils studied were obtained prior to SFCxGC analysis. Tables A1-A5 in Appendix A list all the compounds identified in *Cymbopogon flexuosus*<sup>9</sup> and *citratus*<sup>10</sup>, *Tagetes minuta*<sup>11</sup>, *Artemisia afra*<sup>12</sup> and *Pelargonium captitum*<sup>13</sup>, essential oils from literature. Figure 6.3 shows a two-dimensional separation of *C. flexuosus* oil. As shown earlier with standard terpene hydrocarbons, this group is well separated from the other chemical classes. This group consist of mono-terpenes, (C10 hydrocarbons e.g.  $\alpha$ -pinene, limonene, p-cymene), sesquiterpenes components (C15 hydrocarbons) and diterpenes (C20 hydrocarbons). The next group consists of the carbonyls (with ester, ketones and aldehydes co-eluting) and the last group consist of alcohols co-eluting with the aldehydes (geranial and neral). Figure 6.3 of a *C. flexuosus* essential oil is characterized by two intense peaks (15,16) occurring at the usual region of alcohols in the SFC dimension. These are the two citral isomers (neral and geranial) which are the major components in the oil<sup>9</sup>. We suspect that they undergo keto-enol tautomerism with the enol form stabilized by stronger hydrogen bonding on the silica gel stationary phase. This could account for their added retention as is also the case in SFC silica gel data from literature<sup>16</sup>. We believe this is a

reversible transformation, as apposed to some permanent transformations that have been documented in the chromatographic analysis of essential oils by Sandra and Bicchi<sup>14</sup>: Most artefacts occur in the injector of a gas chromatograph and such reactions are difficult to detect. An example of transformation in the course of an analysis, is the isomerization of germacrene D which is an important constituent found in the essential oil of peppermint<sup>14</sup>. This sesquiterpene hydrocarbon undergoes several rearrangements which can be thermal, photochemical, or acid-catalyzed<sup>14</sup>. Another example is the transformation of linalyl acetate in the essential oil of Petitgrain (contains 80% of linalyl acetate), which at high temperature (>200 °C) converts to  $\beta$ -myrecene (elimination of acetate group) also to limonene, cis- $\beta$ ; trans- $\beta$ , and allo-ocimene<sup>14</sup>.

The use of compound parameters such as Kovats index, polarity and volatility in SFCxGC is demonstrated to identify some of the major peaks contained in the oil of *C. flexuosus*. With this information we can distinguish the different types of terpenes such as monoterpenes, sesquiterpenes and diterpenes in figure 6.3. Some of the tentatively assigned peaks are outlined in table 6.3. Jennings and Shimbato<sup>15</sup> pointed out that retention indices have some value as complementary criterion. It is well known that the use of Kovats indices can facilitate crucial identifications in the case of compounds with similar features such as most mono- and sesqui- terpenes, that have near-identical mass spectra.

A chromatogram of *C. citratus* oil is shown in figure 6.4. It shows very similar patterns of peaks as *Cymbopogon flexuosus* oil. Most of the compounds spread on the two-dimensional plane resemble *Cymbopogon flexuosus* oil obtained in figure 6.3. The common feature about both oils is that they contain two isomers, neral and geranial (cis and trans-citral) as the main constituents<sup>9,10</sup>. This is the source of citral used mainly in perfume industries. Normally neral and geranial should represent about 20 % and 40 % or more of the lemongrass composition. The absence of the unidentified compound marked X in *Cymbopogon citratus* oil also distinguishes it from *C. flexuosus*. Table 6.3 and 6.4 indicate some of the numbered peaks tentatively identified in *Cymbopogon*

*flexuosus* and *C. citratus* oils from the literature list of compounds for the two the oils<sup>9,10</sup>.

Because of the structural similarities and equal molecular mass amongst the terpene hydrocarbons positive identification of these compounds is difficult in one-dimensional separation as already discussed. However, with the use of the advantages of comprehensive two-dimensional SFCxGC to spread compounds over two-dimensions it is much easier to resolve more peaks because of the enhanced peak capacity. We can distinguish the C10, C15 and C20 terpene hydrocarbons because the oxygenated compounds are separated from them. A high degree of order is obtained because of the high polar selectivity offered by silica-gel PLOT SFC separation at lower temperatures and effective volatility analysis by resistively heated GC. Compound identification can more readily be made since the two sets of retention data provide both polarity and volatility information for the sample components.

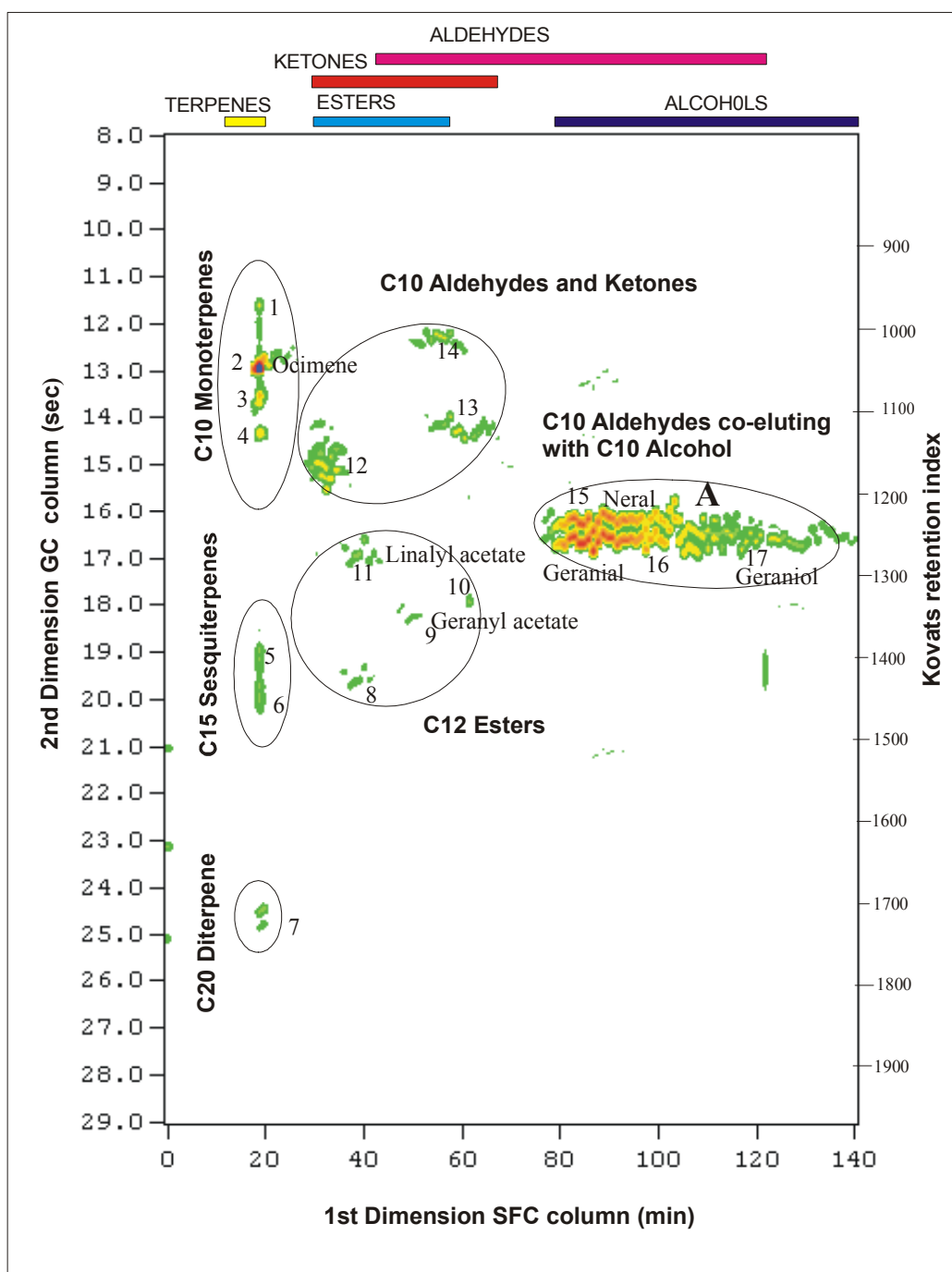
**Table 6.3.** Composition<sup>10</sup>, chemical class, molecular weight, and retention data<sup>14</sup> for *Cymbopogon flexuosus*. (Peak numbers refer to identified peaks in figure 6.3).

*I*=Kovats index on methyl silicone, *T*= isothermal temperature at which the index was determine<sup>14</sup>

Peak No.	Compound <sup>10</sup>	Chemical class	MW	I <sup>14</sup>	T <sup>14</sup>	% <sup>10</sup>
1	$\alpha$ -Pinene	C10 Monoterpene	136	942	100	0.06-2.67
2	<b>Camphene</b>	C10 Monoterpene	136	953	100	<b>0.07-13.46</b>
	$\beta$ -Pinene	C10 Monoterpene	136	978	100	0.1
	Myrcene	C10 Monoterpene	136	988	100	1.93-4.33
	$\alpha$ -Phellandrene	C10 Monoterpene	136	1007	110	0.05-0.16
3	Limonene	C10 Monoterpene	136	1024	100	0.035-3.03
	$\beta$ -Phellandrene	C10 Monoterpene	136	1034	100	0.11-0.40
	(Z)- $\beta$ -Ocimene	C10 Monoterpene	136	1027	100	0.05-0.20
4	<b>(E)-<math>\beta</math>-Ocimene</b>	C10 Monoterpene	136	1042	100	<b>0.82-20.99</b>
	<b><math>\gamma</math>-Terpinene</b>	C10 Monoterpene	136	1056	100	<b>0.21-9.91</b>
	Terpinolen	C10 Monoterpene	136	1074	100	0.10-0.43
14	Citronellal	C10 Aldehyde	154	1143	135	0.06-0.18
	Linalool	C10 Alcohol	154	1097	135	0.77-9.95
15	<b>Neral</b>	C10 Aldehyde	152	1227	120	<b>1.84-10.42</b>
16	<b>Geranial</b>	C10 Aldehyde	152	1260	120	<b>1.82-15.03</b>
	$\alpha$ -Terpineol	C10 Alcohol	154	1178	135	0.06-1.42
	Borneol	C10 Alcohol	154	1177	175	0.28-4.86
9	Geranyl acetate	C12 Ester	196	1363	135	0.62-7.74
11	Linalyl acetate	C12 Ester	196	1240	130	2.3
	Nerol	C10 Alcohol	154	1218	120	0.14-0.32
17	<b>Geraniol</b>	C10 Alcohol	154	1234	175	<b>3.0-74.72</b>

**Reference:** <sup>14</sup> N.W. Davies, *J. Chromatogr.*, 503(1990) 1-24, for Kovats retention indices.

<sup>10</sup> Weiss, *Essential oil Crop*, CAB International, 1977 (for essential oil composition and percentage amount of each compound present)



**Figure 6.3.** SFCxGC chromatogram of *Cymbopogon flexuosus* oil. SFC<sub>PLOT</sub> pressure ramp 110-200 atm at 1 atm/min, temperature =28 °C, Modulation: 5 s collection time and equilibration time = 5 s, Fast GC ramped (-50 to 300 ) °C at 450 °C/min. Scale for Kovats index (1.78 sec = 100 units) calculated from peak 2 (Camphene, KI = 956) and peak 15 (Neral, KI =1227)

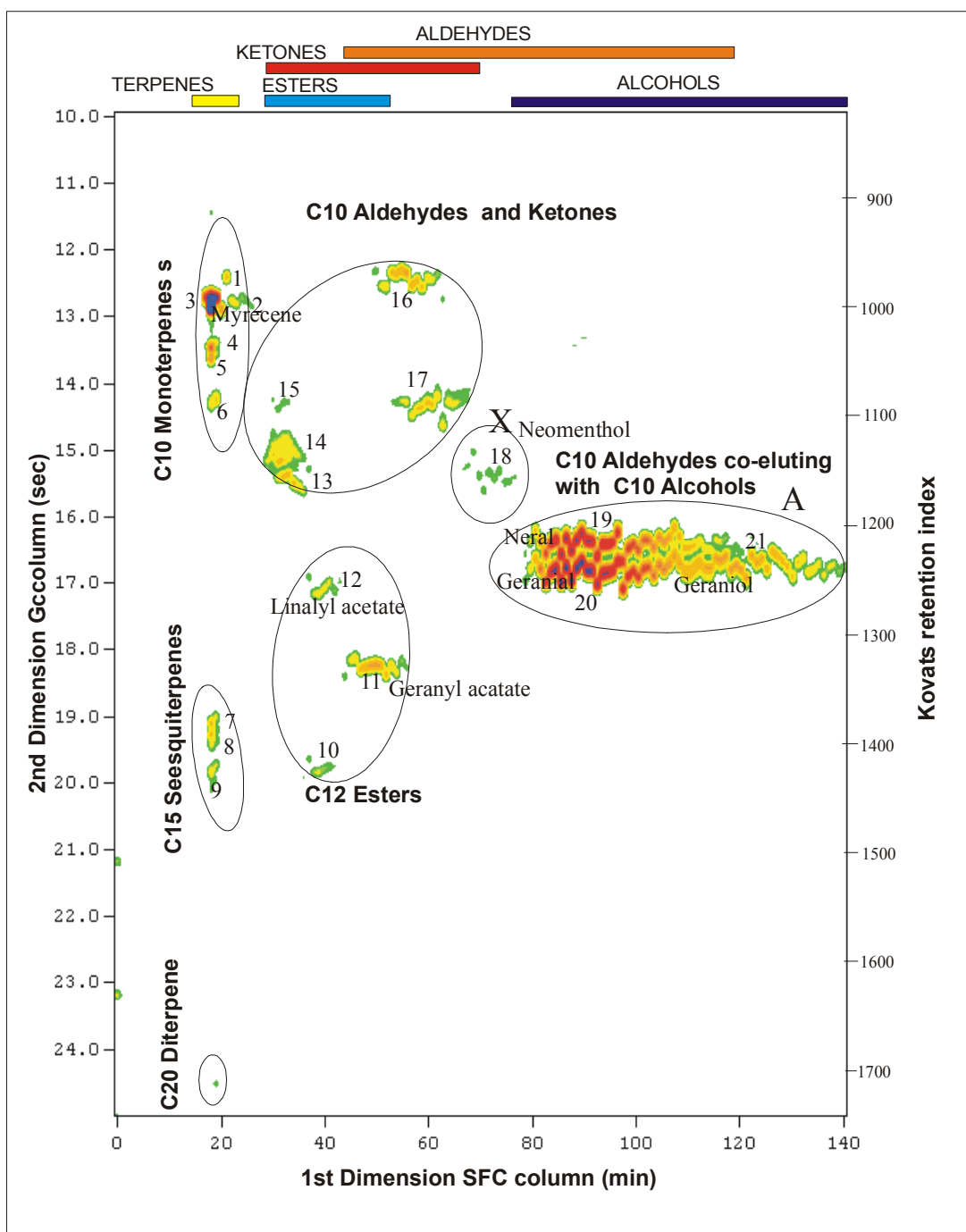
**Table 6.4.** Composition<sup>10</sup>, chemical class, molecular weight, and retention data<sup>14</sup> for *Cymbopogon citratus*. (Peak numbers refer to identified peaks in figure 6.4)

*I*=Kovats index on methyl silicone, *T*= isothermal temperature at which the index was determine<sup>14</sup>

Peak No.	Compound <sup>10</sup>	Chemical class	MW	I <sup>14</sup>	T <sup>14</sup>	% <sup>10</sup>
1	2,6-Dimethyloctane	C10 Monoterpene	142	938	100	0.1
3	<b>Myrcene</b>	C10 Monoterpene	136	988	100	<b>24.3</b>
5	(Z)- $\beta$ -Ocimene	C10 Monoterpene	136	1027	100	1.0
6	(E)- $\beta$ -Ocimene	C10 Monoterpene	136	1042	100	0.7
4	p-Cymene	C10 Monoterpene	134	1016	100	0.5
	trans-Allo-Ocimene	C10 Monoterpene	136	1120	110	0.1
16	Fenchone	C10 Ketone	136	1077	105	0.2
9	$\beta$ -Caryophyllene	C15 Sesquiterpene	204	1436	150	0.3
	Tetrahydrolinalool	C10 Alcohol	136	1088	90	0.2
16	Citronellal	C10 Aldehyde	154	1143	135	0.3
	$\beta$ -Patchoulene	C15 Sesquiterpene		1378	120	0.2
	Linalool	C10 Alcohol	154	1097	135	0.6
14	Camphor	C10 Ketone	154	1126	110	0.1
18	<b>Neomenthol</b>	C10 Alcohol	156	1159	120	<b>3.3</b>
	Terpinen-1-ol	C10 Alcohol	154			0.4
12	<b>Linalyl acetate</b>	C12 Ester	196	1240	130	<b>2.3</b>
11	Geranyl acetate	C12 Ester	196	1363	135	
20	<b>Geranial</b>	C10 Aldehyde	152	1260	120	<b>33.7</b>
	Sabinol	C10 Alcohol	152	1224	175	0.1
	Nerol	C10 Alcohol	154	1218	120	0.8
21	Geraniol	C10 Alcohol	154	1234	175	1.9

**Reference**<sup>14</sup> N.W. Davies, *J. Chromatogr.*, 503(1990) 1-24, for Kovats retention indices.

<sup>10</sup>A Weiss, *Essential oil Crop*, CAB International, 1977 (for essential oil composition and percentage amount of each compound present)



**Figure 6.4.** SFCxGC chromatogram of *Cymbopogon citratus* oil. SFC<sub>PLOT</sub> pressure ramp 110-200 atm at 1 atm/min, temperature =28 °C, Modulation : 5 s collection time and equilibration time = 5 s, Fast GC ramped (-50 to 300 °C) at 450 °C/min. Scale for Kovats index (1.51 sec = 100 units) calculated from peak 3 (Myrcene, KI = 988) and peak 19 (Neral, KI =1227)

Figure 6.5 represents a chromatogram of a *Pelargonium capitatum* essential oil. The chemical compounds of the ester group are dominating in the oil. Some of the major oil components identified using the chemical standards and knowledge of the compound volatility and polarity are summarized in Table 6.5. Figure 6.6 illustrates a typical SFCxGC chromatogram of *Tagetes minuta* essential oil, while the chromatogram of *Artemisia afra* oil is presented by Figure 6.7. Table 6.6. presents some of the identified components of *Tagetes minuta* oil. Dihydrotagetone, (E)-tagetone, (Z)- $\alpha$ -Ocimene are the main constituent components of the *Tagetes minuta* oil. Qualitatively, several differences were observed between the four essential oils (*Pelargonium radens X capitatum*, *Cymbopogon*, *Tagetes minuta* and *Artemisia afra*).

By using the distinctive peak patterns observed in all four essential oil chromatograms (Figure 6.3 to 6.7), some of the components such as linalool and geraniol identified in *C. citratus* & *flexuosus* are detectable in *Pelargonium radens X capitatum* oil. A striking differences among these oils is that *Pelargonium capitatum* is rich with sesquiterpenes, alcohols, C11, C12, and C14 esters and *Cymbopogon* oils contain terpenes and aldehydes. *Tagetes minuta* and *Artemisia afra* are both rich with carbonyls and *Tagetes minuta* oil (Figure 6.6) shows a detectable alcohol component. Furthermore, more carbonyl compounds are detectable in *Pelargonium capitatum* and *Tagetes minuta* oils than the other oils. These characteristic features provide a means of differentiating the four oils and useful conclusions can readily be drawn about the type of oil based on these analytical measurements.

Comparison of figure 6.5 to 6.3 and 6.4 immediately reveals some of the advantages of two-dimensional plane chromatograms developed by SFCxGC analysis. *Pelargonium* oil (figure 6.5) shows the presence of the most ester compounds compared to the *Cymbopogon flexuosus* and *citratus* oils (figure 6.3 and 6.4). The presence of the peaks marked XZ in *Pelargonium* oil at the alcohols region in SFC dimension and esters (C13 & C14) in fast GC scale differentiate *Pelargonium* from *Cymbopogon*. The peaks marked XZ can be assumed to be C15 alcohols.



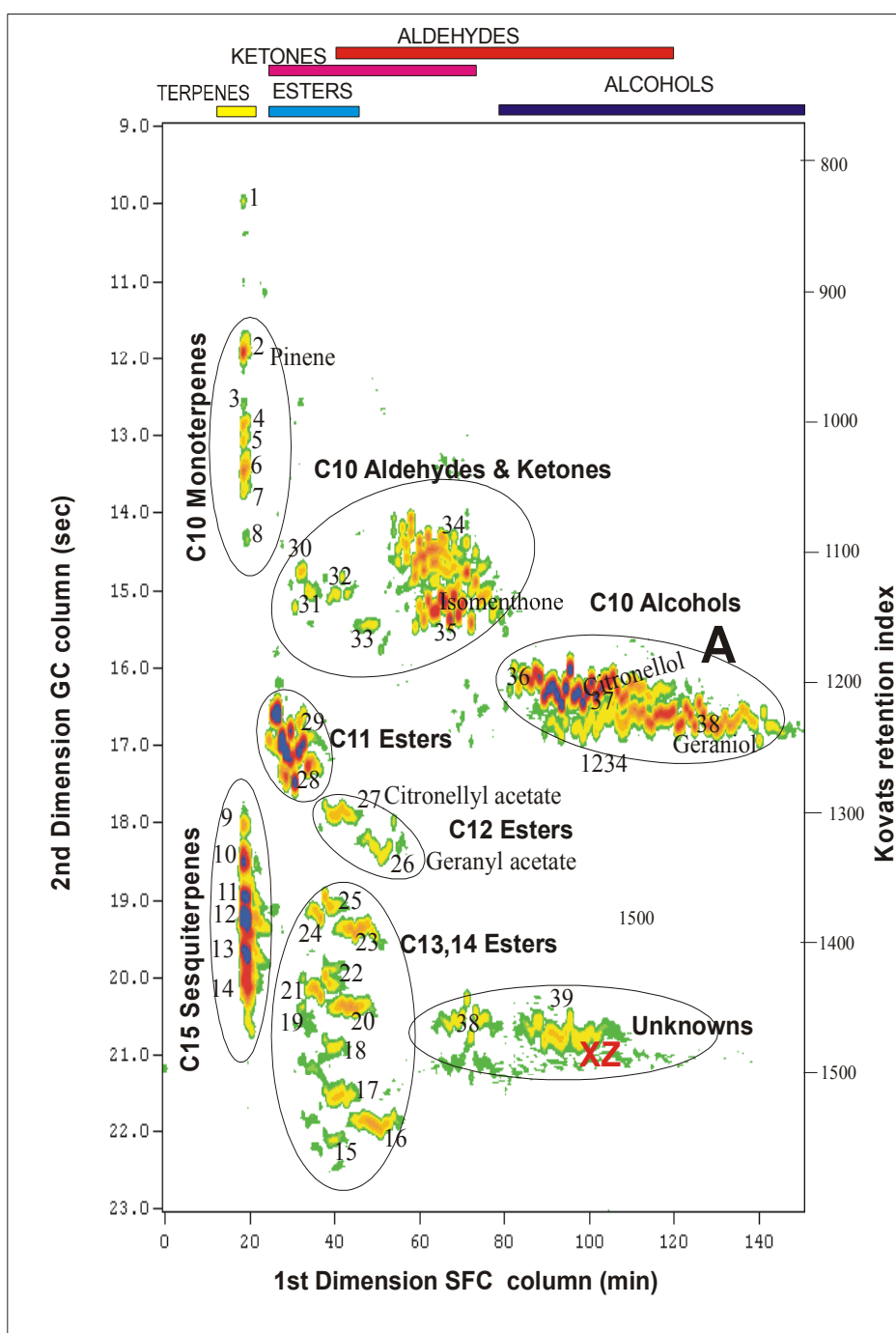
**Table 6.5.** Composition<sup>10</sup>, chemical class, molecular weight, and retention data<sup>14</sup> for *Pelargonium capitatum* l. (Peak numbers refer to identified peaks in figure 6.5).

*I*=Kovats index on methyl silicone, *T*= isothermal temperature at which the index was determined, or "prog" if the index was determined using temperature programming<sup>14</sup>

Peak No.	Compound	Chemical class	MW	I <sup>14</sup>	T <sup>14</sup>	% <sup>10</sup>
1	$\alpha$ -Pinene	C10 Monoterpene	136	942	100	1.00
2	Myrcene	C10 Monoterpene	136	988	100	0.30
3	Cis- $\beta$ -Ocimene	C10 Monoterpene	136	1027	100	0.30
4	Cis-Rose oxide	C10 Oxygenate		1087	prog	0.20
5	trans-Rose oxide	C10 Oxygenate		1100	prog	Ng
<b>36</b>	<b>Linalool</b>	C10 Alcohol	154	1097	135	<b>4.60</b>
	Menthone	C10 Ketone	154	1158	130	0.40
<b>35</b>	<b>Isomenthone</b>	C10 Ketone		1156	130	<b>7.80</b>
	$\alpha$ -Terpineol	C10 Alcohol	154	1178	135	0.30
<b>37</b>	<b>Citronellol</b>	C10 Alcohol	156	1224	175	<b>19.00</b>
<b>38</b>	<b>Geraniol</b>	C10 Alcohol	154	1234	175	<b>21.50</b>
	Geranial	C10 Aldehyde	152	1260	120	Ng
	<b>Citronellyl formate</b>	C11 Ester	184	1261	prog	<b>8.50</b>
	<b>Geranyl formate</b>	C11 Ester	182	1282	prog	<b>9.50</b>
26	Geranyl acetate	C12 Ester	196	1363	prog	
27	Citronellyl acetate	C12 Ester	196	1335	135	0.50
10	$\beta$ -Bourbonene	C15 Sesquiterpene		1406	prog	0.70
11	$\beta$ -Caryophyllene	C15 Sesquiterpene	204	1428	prog	0.80
	Citronellyl propionate	C13 Ester		1427	prog	0.20
<b>12</b>	<b>Guaiadiene 6.9</b>	C15 Sesquiterpene				<b>7.20</b>
	Geranyl propionate	C13 Ester				1.60
13	Germacene D	C15 Sesquiterpene	204	1488	150	2.30
	Citronellyl butyrate	C14 Ester	226	1511	prog	1.00
	Geranyl butyrate	C14 Ester	224	1532	prog	1.20
	Phenylethyl tiglate	C14 Ester				0.70
	Citronellyl tiglate	C14 Ester				0.10
	Geranyl tiglate	C14 Ester				1.30

**Reference**<sup>14</sup> N.W. Davies, *J. Chromatogr.*, 503(1990) 1-24, for Kovats retention indices.

<sup>10</sup> A Weiss, *Essential oil Crop*, CAB International, 1977 (for essential oil composition and percentage amount of each compound present)



**Figure 6.5.** SFCxGC chromatogram of *Pelargonium* essential oil SFC<sub>PLOT</sub> pressure ramp 110-200 atm at 1 atm/min, temperature =28 °C, Modulation: 5 s collection time and 5 s equilibration, Fast GC ramped (-50 to 300) °C at 450 °C/min. Scale for Kovats index (1.64 sec = 100 units) calculated from peak 2 (Pinene, KI = 942) and peak 38 (Geraniol, KI =1234).

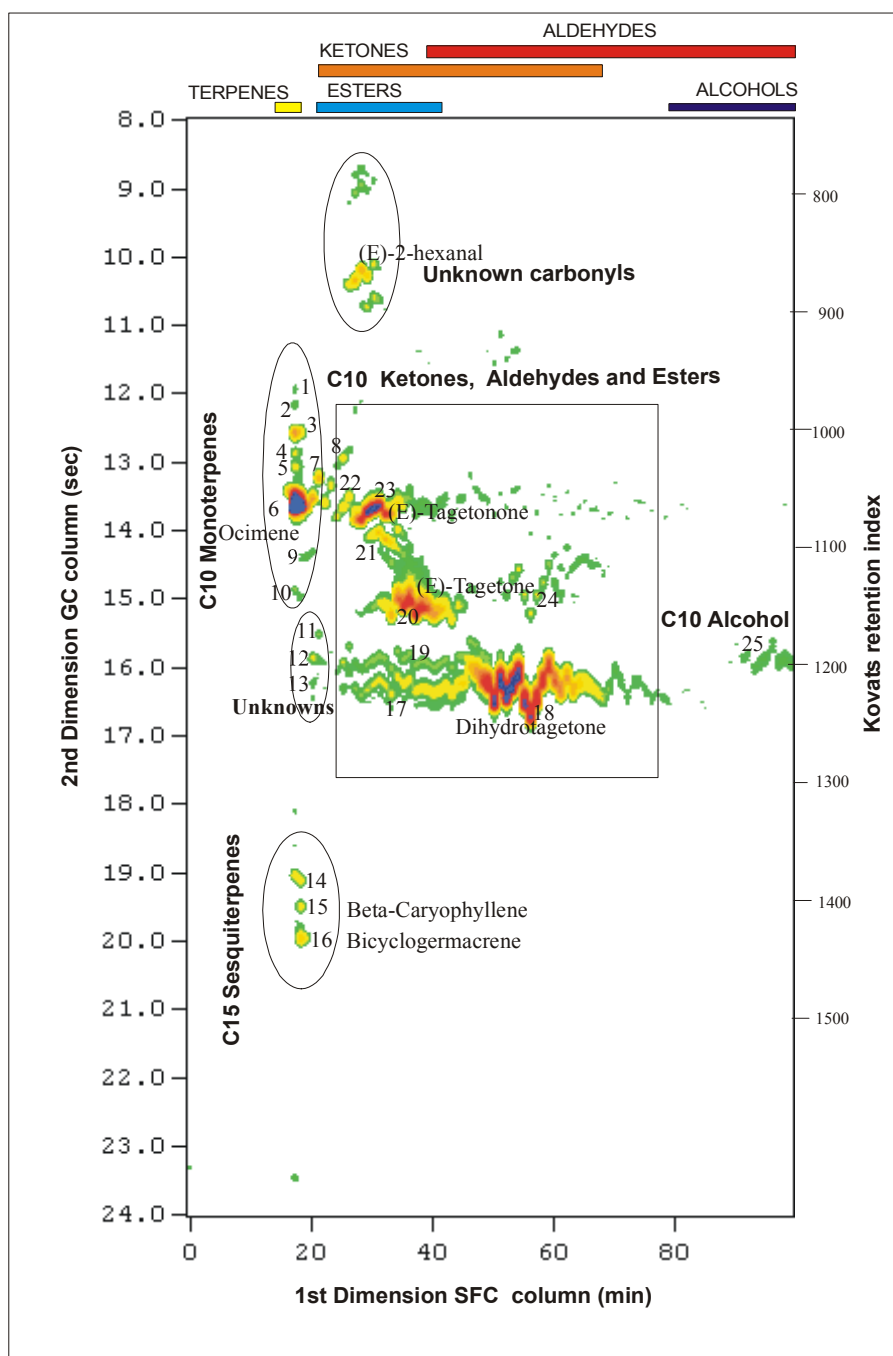
**Table 6.6.** Composition<sup>11</sup>, chemical class, molecular weight, and retention data<sup>14</sup> for *Tagetes minuta*. (Peak numbers refer to identified peaks in figure 6.6)

*I*=Kovats index on methyl silicone, *T*= isothermal temperature at which the index was determined, or "prog" if the index was determined using temperature programming<sup>14</sup>

Peak No.	Compound <sup>11</sup>	Chemical class	MW	I <sup>14</sup>	T <sup>14</sup>	% <sup>11</sup>
1	$\alpha$ -Pinene	C10 Monoterpene	136	942	100	0.06
	Ethyl-2-methylbutyrate	C10 Monoterpene	136			0.08
	Sabinene	C10 Monoterpene	136	972	100	0.96
	Myrcene	C10 Monoterpene	136	988	100	0.1
	$\alpha$ -Phellandrene	C10 Monoterpene	136	1007	110	0.09
	$\alpha$ -Terpinene	C10 Monoterpene	136	1016	100	0.02
3	Limonene	C10 Monoterpene	136	1025	100	7.24
	$\beta$ -Phellandrene	C10 Monoterpene	136	1007	110	0.07
	(E)-2-hexanal	C6 Aldehyde				0.06
6	<b>(Z)-<math>\beta</math>-Ocimene</b>	C10 Monoterpene	136	1027	100	<b>28.49</b>
	$\gamma$ -Terpinene	C10 Monoterpene	136	1056	100	0.05
	(E)- $\beta$ -Ocimene	C10 Monoterpene	136	1042	100	0.39
	allo-Ocimene	C10 Monoterpene	136	1132	Prog	0.32
15	$\beta$ -Caryophyllene	C15 Sesquiterpene	204	1428	Prog	0.47
16	Bicyclogermacrene	C15 Sesquiterpene	204	1490	Prog	0.1
18	<b>Dihydrotagetone</b>	C10 Ketone				<b>30.3</b>
	(Z)-Tagetone	C10 Ketone				0.25
	Decanal	C10 Aldehyde				0.12
23	(E)-Tagetone	C10 Ketone				4.8
	(Z)-Tagetonone	C10 Ketone				1.87
20	<b>(E)-Tagetonone</b>	C10 Ketone				<b>15.35</b>
	Iso-piperitenone	C10 Ketone				0.26

**Reference** <sup>14</sup> N.W. Davies, *J. Chromatogr.*, 503(1990) 1-24, for Kovats retention indices.

<sup>11</sup> J. Chalchat, R.P. Granny, A. Muhayima, *J. Essent. Oil Re.*, 7(1995)375-386 (for essential oil composition and percentage amount of each compound present)



**Figure 6.6.** SFCxGC chromatogram of *Tagetes minuta* essential oil. SFC<sub>PLOT</sub> pressure ramp 110-200 atm at 1 atm/min, temperature = 28°C, Modulation, 5 s collection time and equilibration time = 5 s, Fast GC ramped -50 to 300 °C at 450 °C/min. Scale for Kovats index based on retention time scale of figure 6.5 (*Pelargonium* oil)

**Table 6.7.** Composition<sup>12</sup>, chemical class, molecular weight, and retention data<sup>14</sup> for *Artemisia afra* oil. (Peak numbers refer to identified peaks in figure 6.7)

*I*=Kovats index on methyl silicone, *T*= isothermal temperature at which the index was determined, or "prog" if the index was determined using temperature programming<sup>14</sup>

Peak No.	Compound <sup>12</sup>	Chemical class	MW	I <sup>14</sup>	T <sup>14</sup>	% <sup>12</sup>
3	Tricyclene	C10 Cycloalkane		928	100	0.1-0.2
4	$\alpha$ -Pinene	C10 Monoterpene	136	939	100	0.4-1.1
6	$\alpha$ -Fenchene	C10 Monoterpene	136	957	110	0.1-1.0
5	<b>Camphene</b>	C10 Monoterpene	136	956	100	<b>0.3-3.9</b>
7	$\beta$ -Pinene	C10 Monoterpene	136	978	100	0.1-0.7
8	Sabinene	C10 Monoterpene	136	976	Prog	0.1-1.1
9	Myrcene	C10 Monoterpene	136	984	100	0.1-1.1
	$\alpha$ -Terpinene	C10 Monoterpene	136	1016	100	0.1-1.1
	Dehydro-1,8-cineol	C10 Alcohol				0.1-0.2
	Limonene	C10 Monoterpene	136	1025	100	0.1-0.2
	<b>1.8-Cineol</b>	C10 Alcohol	136	1025	100	<b>0.1-27.9</b>
	(E)- $\beta$ -Ocimene	C10 Monoterpene	136	1027	100	0.1-0.3
	$\gamma$ -Terpinene	C10 Monoterpene	136	1056	100	0.3-1.9
	p-Cymene	C10 Monoterpene	136	1018	100	0.3-2.0
	Terpinolene	C10 Monoterpene	136	1081	100	0.1-0.5
	$\beta$ -Caryophyllene	C15 Sesquiterpene	204	1432	150	0.5-0.2.3
27	<b>Artemisia ketone</b>	C10 Ketone	152	1153	Prog	<b>6.3-41.9</b>
	<b>Santolina alcohol</b>	C10 Alcohol				<b>3.1-10.1</b>
	$\alpha$ -Thujone	C10 Ketone		1100	110	1.0-2.9
	Artemisyl acetate	C12Ester				0.1
	$\beta$ -Thujone	C10 Ketone				Trace
	Artemisia alcohol	C10 Alcohol				0.1
	cis-Sabinene hydrate					0.2-0.6
17	<b><math>\alpha</math>-Copaene</b>	C15 Sesquiterpene		1398	Prog	<b>8.5-27.1</b>
	trans-Sabinene hydrate					1.8-4.4
	cis-p-Mentha-2-en-1-ol	C10 Alcohol		1111	Prog	0.2-0.4
	Bornyl acetate	C12Ester		1278	135	0.3-1.5
	Terpinen-4-ol	C10 Alcohol		1129	135	0.1
	Myrtenal	C10 Aldehyde		1173	120	0.1
	trans-p-Mentha-2-en-1-ol	C10 Alcohol		1128	Prog	0.2-0.3
	<b>Borneol</b>	C10 Alcohol		1154	110	<b>0.6-3.4</b>
	$\alpha$ -Terpineol	C10 Alcohol		1178	135	0.1-.2.5

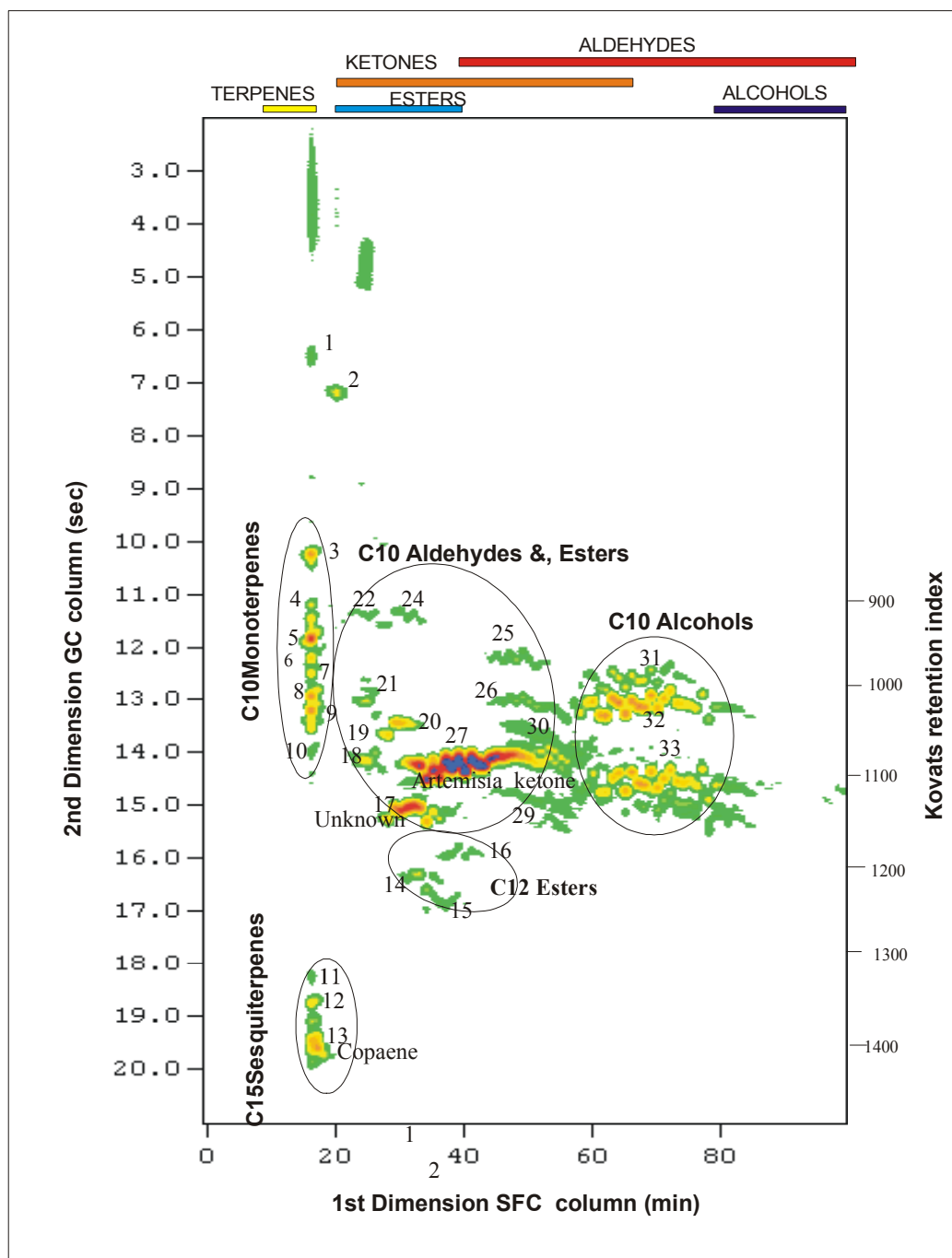
**Reference**<sup>14</sup> N.W. Davies, *J. Chromatogr.*, 503(1990) 1-24, for Kovats retention indices.

<sup>12</sup> JS Chagonda, C. Makanda, J. Claude Chalchat, *Flavour and Frag. J.*, 14(1999) 140-142 (for essential oil composition and percentage amount of each compound present)

**Table 6.7.** Composition<sup>12</sup>, chemical class, molecular weight, and retention data<sup>14</sup> for *Artemisia afra* oil. (Peak numbers refer to identified peaks in figure 6.7)

*I*=Kovats index on methyl silicone, *T*= isothermal temperature at which the index was determined, or "prog" if the index was determined using temperature programming<sup>14</sup>

Peak No.	Compounds	Chemical class	MW	I <sup>14</sup>	T <sup>14</sup>	% <sup>12</sup>
11	Bicyclogermacrene	C15 Sesquiterpene	204	1490	Prog	0.2-0.5
	Piperitol	C10 alcohol				0.1-.07
13	δ-Cadinene	C15 Sesquiterpene	204	1507	130	0.5-0.8
	Cuminaldehyde	C10 Aldehyde				0.5
	Myrtenol	C10 Alcohol	152	1281	120	0.1
12	Calamenene	C15 Sesquiterpene	204	1502	Prog	0.1-0.9
	cis-Carveol	C10 Alcohol		1215	120	0.1
	trans-Caryophyllene oxide	C15 ether		1576	Prog	0.1
	Methyl linolenate					0.1
	Germacene-D-4-ol	C15 Alcohol				0.1
	p-Cymen-8-ol	C10 Alcohol	152	1167	115	0.1
	Spathulenol	C10 Alcohol				0.1
	T-muurolol	C10 Alcohol				0.5
	Intermomedol	C10 Alcohol				0.4



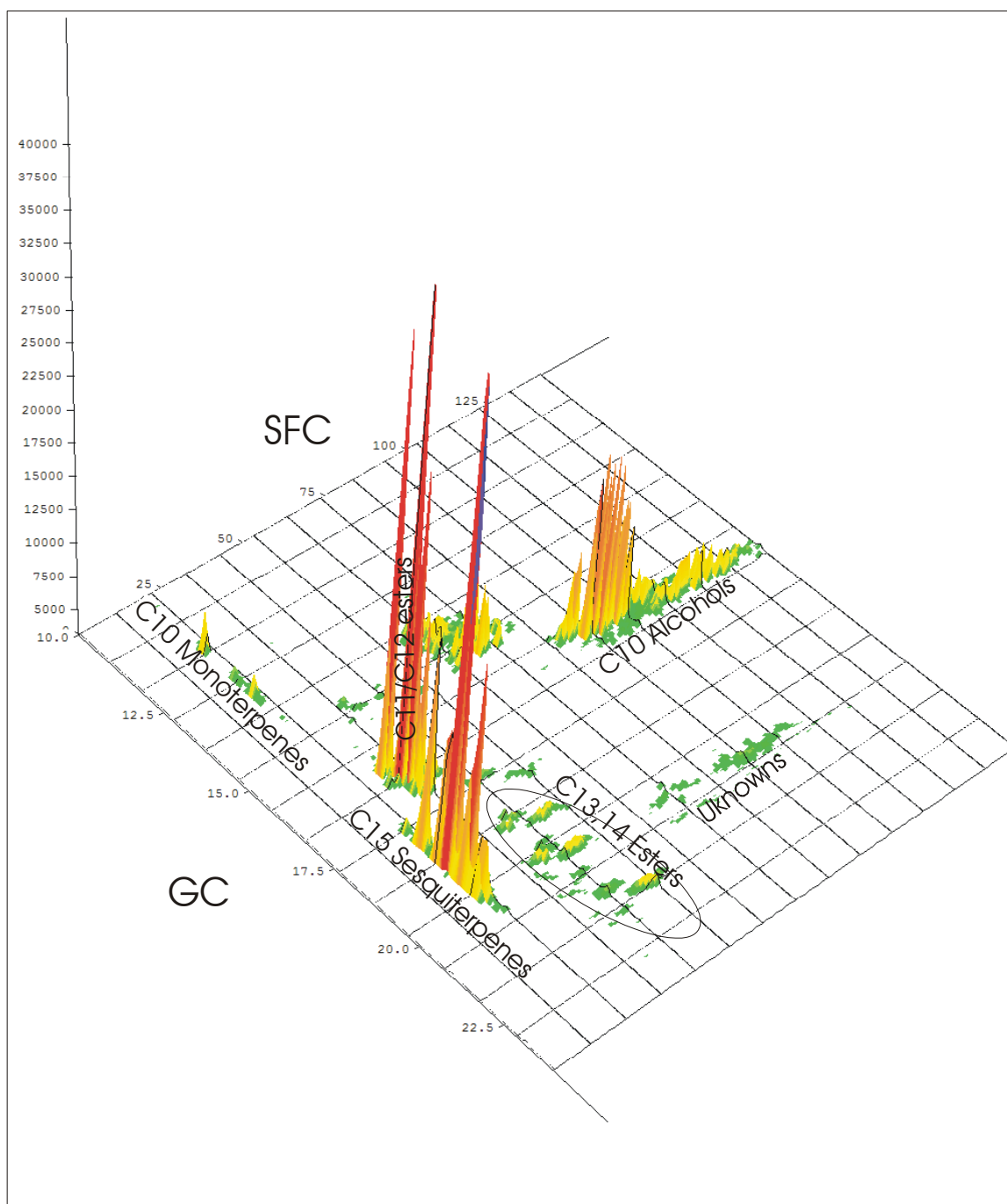
**Figure 6.7.** SFCxGC chromatogram of *Artemisia afra* essential oil. SFC<sub>PLOT</sub> pressure ramp 110-200 atm at 1 atm/min, temperature = 28°C, Modulation :5 s collection time and equilibration time = 5 s, Fast GC ramped -50 to 300 °C at 450 °C/min. Scale for Kovats index based on retention time scale of figure 6.5 (*Pelargonium* oil)

All these oils were run at identical chromatographic condition and comparison of the oils is easily achieved by doing peak matching with identification of similar regions in the essential oil chromatograms. For true visualization of very small co-eluting peaks, that are not visible in the 2D-plane, 3D-plane structure gives a clear picture of the peaks especially for the low concentration components. Figure 6.8 shows a typical three-dimensional plane chromatogram of the comprehensive SFCxGC analysis of *Pelargonium capitatum X randens* essential oil.

It is easier to identify some of the single components separated on the essential oil samples by comparing their peak retention times with the ones of the chemical standards for individual peak identification. Therefore, comparing the *Pelargonium capitatum X radens* essential oil in figure 6.5 with the essential oil standard chromatogram (Figure 6.2), it is evident that terpineol, geraniol and linalool are some of the last eluting alcohol compounds in *Pelargonium* oil.

When displaying the chromatogram as a contour plot, within the two-dimensional plane of the two retention time axes, compounds are ordered according to their chemical or molecular functionality, structure or shape, which makes verification of the compounds relatively easy and reliable for SFCxGC, provided the composition of the sample is known from other studies (e.g. GC-MS). Two types of separation can be performed, namely, a group-type-separation and a separation of target compounds. Compounds of a particular chemical class will have comparable first dimension retention times, and are grouped together in bands along a one-dimensional plane. The identification of compounds ordered within these bands is therefore simplified by using fast GC.





**Figure 6.8** SFCxGC Three dimensional Chromatogram of *Pelargonium* essential oil.

### 6.3.3 Qualitative comparison of four *Cymbopogon citratus* oil samples

The quality of essential oils varies with place of origin, climate, etc. One of the objectives of this research was to evaluate the SFCxGC system for fingerprinting of essential oils in order to qualitatively differentiate oils of the same family (e.g. lemongrass) from different places. For this purpose four *cymbopogon citratus* oil samples from different locations (4,5,6,7) were analysed on the SFCxGC system using the same chromatographic conditions already outlined in section 6.3.1.1.

#### 6.3.3.1 Results and Discussions

Figures 6.9 and 6.10 show the comparison of four lemongrass oil samples (4,5,6,7) analysed with SFCxGC. Four peaks marked A to D are highlighted in table 6.8 to show that *Cymbopogon* oil samples differ, with detection of compounds in some oils but not in others. Even in its prototype form, SFCxGC clearly can provide valuable information. Peaks integration facilities, to quantify components in the mixture, can only improve on this fingerprinting ability

**Table 6.8** Qualitative comparison of four lemongrass *citratus* oil samples

<b>Description</b>	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>
lemongrass 4	a	p	p	P
lemongrass 5(nduva)	p	p	p	A
lemongrass 6	a	p	p	P
lemongrass 7	p	p	a	A

Bold capital letter case (**A**, **B**, **C**, **D**)= represent chosen regions in the chromatograms for comparison of the oil sample.

Small letter case: a= absent, p=present

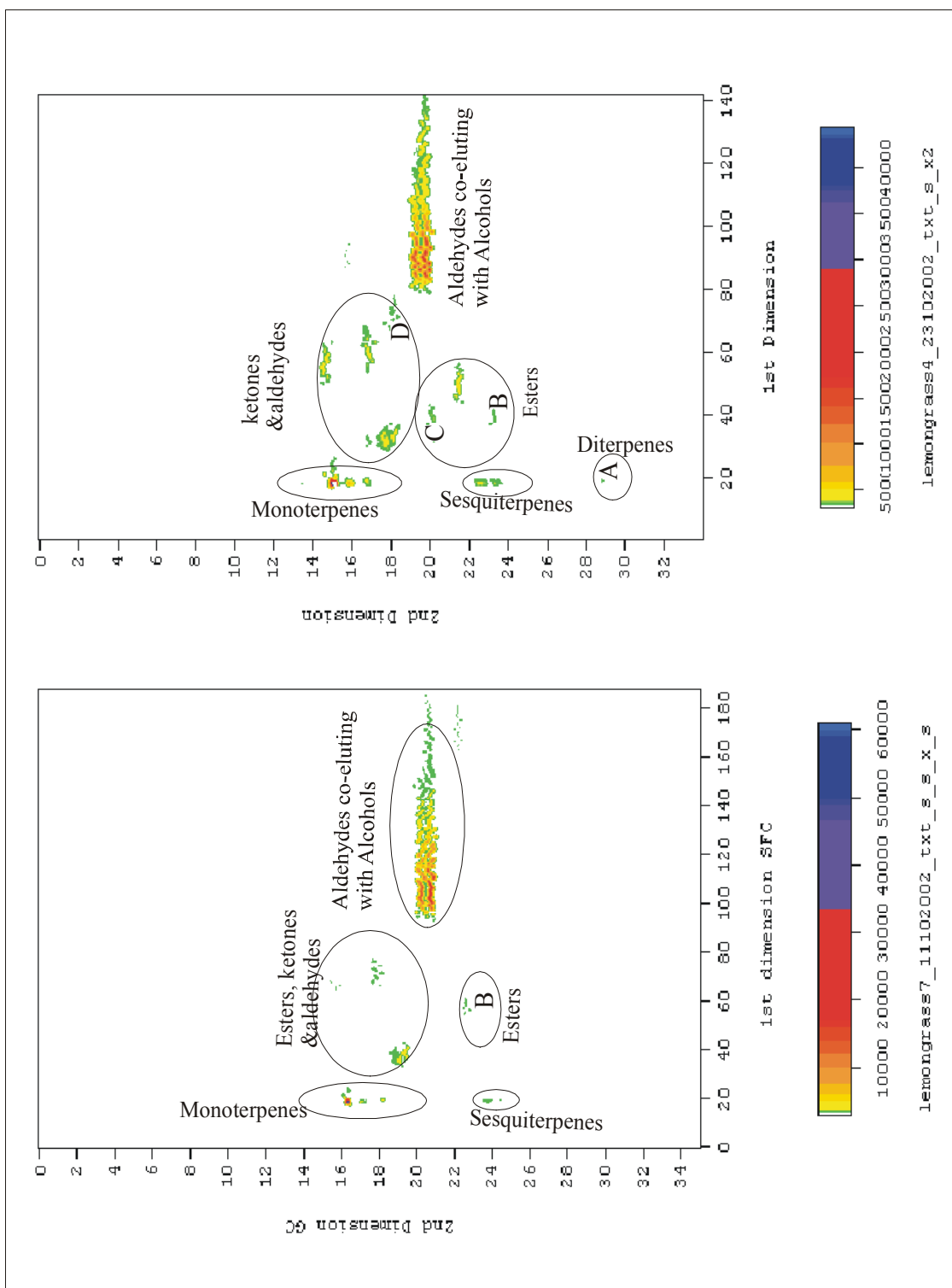


Figure 6.9 SFCxGC chromatogram showing comparison of lemongrass oil samples (4 and 5)

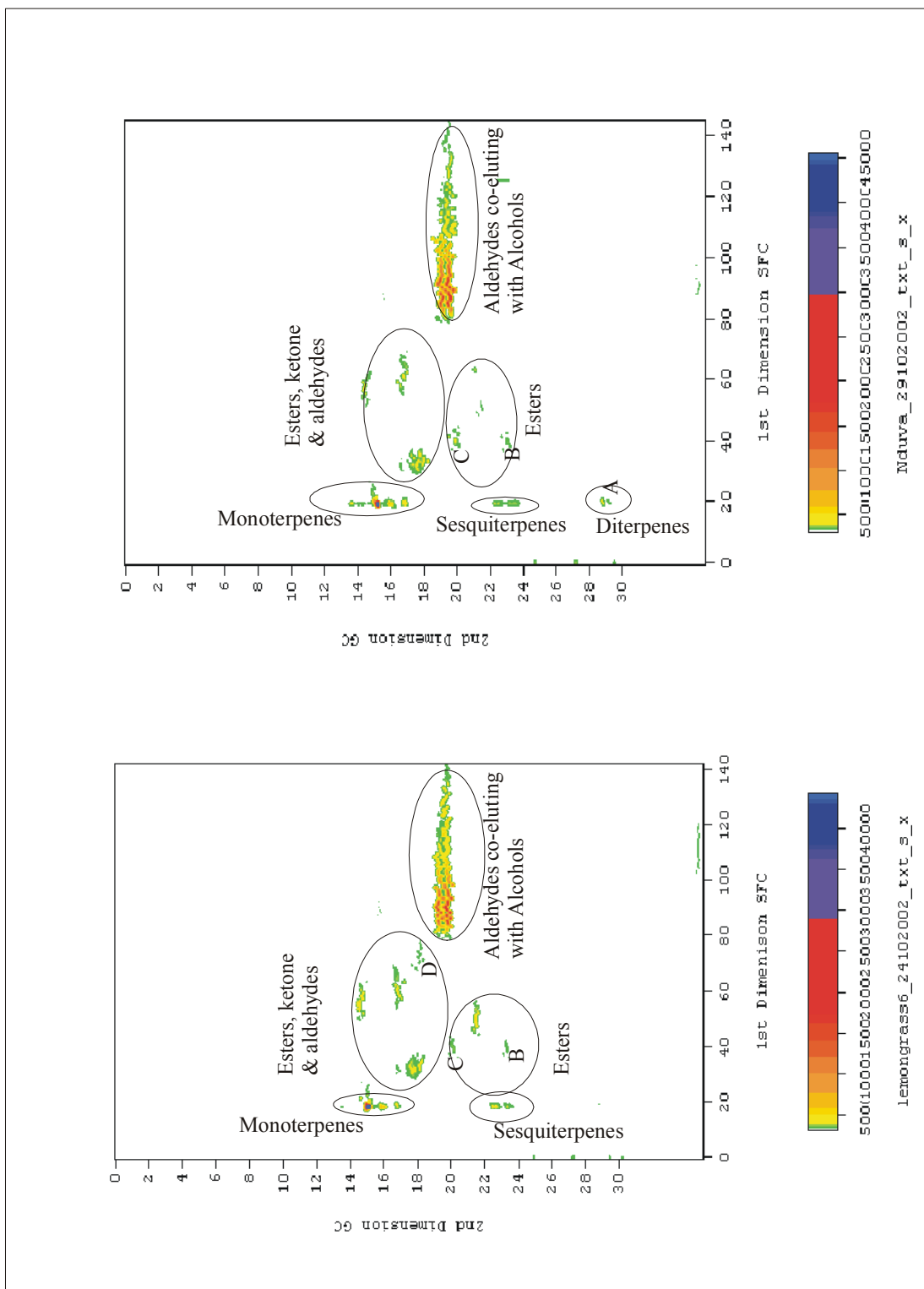


Figure 6.10 SFCxGC chromatogram showing comparison of lemongrass oil samples (6 and 7)

### 6.3.4 Reproducibility of SFCxGC runs

An instrument can only provide reliable information if its analytical data is reproducible. Statistical parameters such as relative standard deviation (%RSD) and standard deviation (SD) are often used to interpret the reproducibility data. For this reason one of the lemongrass oil samples *C. citratus* (no.4) was chosen to do repeated SFCxGC runs.

#### 6.3.4.1 Result and Discussion

The chromatographic conditions outlined in section 6.3.1.1 were used for SFCxGC analysis. A single set of restrictors to control the SFC flow was used. Table 6.9 shows the reproducibility from consecutive lemongrass samples no. 4 (*citratus*) with SFCxGC (accompanying chromatograms in Appendix B, figure (B.1-B.4)). The reproducibility obtained was fair in the first dimension (SFC). The relative standard deviation (RSD) of 6.16 and standard deviation of 2.35 were obtained for the SFC analysis. In Table 6.9 (A7, Appendix A) there is a shift of retention times for peaks (1-3) although they belong to the same group or fraction of terpene hydrocarbons. This retention variability seems to be caused by (1) variation in the linear velocity of the mobile phase and (2) insufficient temperature control of the SFC column at the relatively low temperature (28 °C) by the GC oven.

The second dimension (resistively heated GC) retention of individual peaks shows good reproducibility in table 6.9. With repeated SFCxGC runs the %RSD of 1.94 and SD of 0.34 s were obtained. It is important to point out that the same thermocouple was used for all runs to test the SFCxGC reproducibility. It can be concluded that the results of the present consecutive runs on essential oil sample clearly indicate the necessity of introducing important improvements in the SFC dimension. An improved means to control stable temperature conditions and proper control of the column linear velocity (i.e. pump pressure and restrictor flow) is required to better reflect the true SFCxGC

capability. However, the reproducible retention of peaks in the second dimension under ideal circumstances is impressive.

**Table 6.9** Reproducibility results of SFCxGC runs *C. citratus*.

Peak No.	1st Dimension retention			2nd Dimension retention		
	Mean (min)	SD (min)	%RSD	Mean (sec)	SD (sec)	%RSD
1	20.20	1.31	6.57	14.91	0.02	0.15
2	20.64	1.30	6.28	15.59	0.25	1.59
3	20.38	1.26	6.18	16.52	0.11	0.68
5	42.43	1.87	4.40	22.93	0.13	0.57
6	44.58	3.08	6.90	20.46	0.66	3.24
7	35.90	2.72	7.57	17.99	0.16	0.78
9	66.57	3.77	5.66	17.25	0.75	4.31
8	61.49	3.49	5.71	14.89	0.63	4.21
<b>Average</b>		<b>2.35</b>	<b>6.16</b>		<b>0.34</b>	<b>1.94</b>

### 6.3.5 Effect of the thermocouple on SFCxGC separation

Although SFCxGC analysis provides enough information by spreading compounds on the two-dimensional chromatogram, the appearance of the peaks in the SFCxGC chromatogram depends on the result of the reproducibility of the fast GC retention times. To achieve reproducible retention times of the consecutive runs successfully, special care is required of the thermocouple placement onto the column. Further, direct electrical contact of the thermocouple with the column is not allowed with the present control electronics. Very slow thermal response when the glue droplet is too big (when the thermocouple is placed a fraction of a millimeter away from the column) results in irreproducible retention times. Eventually the ramp program results in an oscillating temperature and the temperature set-point is not well followed.

#### 6.3.5.1 Results and discussions

Figure 6.11 shows a chromatogram of *Tagetes minuta* oil obtained with a good placement of the thermocouple on the column. This implies that the thermal contact between the column and the thermocouple is good and the resulting PID control of the temperature ramp is shown in the one in figure 6.13. Figure 6.14 shows the typical ramp obtained with a bad thermocouple placement on the column. The resulting temperature ramp is not smoothly followed as compared to the one in figure 6.13.

Figure 6.12 is a typical chromatogram obtained with a bad thermocouple placement. All individual components separated in Figure 6.11 are merged into big clusters in Figure 6.12. They are all compressed in the second dimension axis (fast GC) as one broad band. Although most of the information about individual components is lost, chemical class separation obtained in the first dimension is still maintained. Special care is needed to obtain individual compound separation and good reproducible results since bad thermocouple placement can influence the final SFCxGC chromatogram.

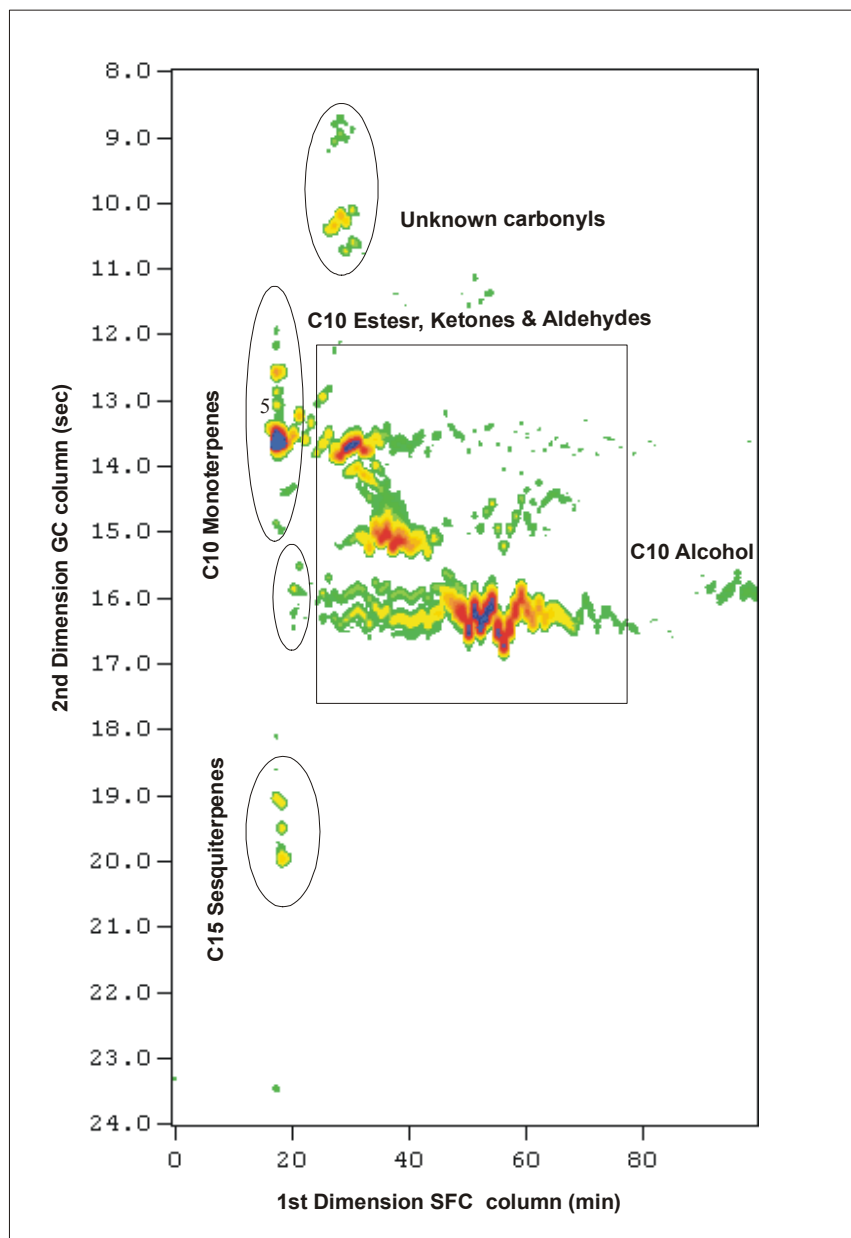


Figure 6.11 SFCxGC *Tagetes minuta* oil with a good thermocouple contact on a 1 m stainless steel, GC capillary column.



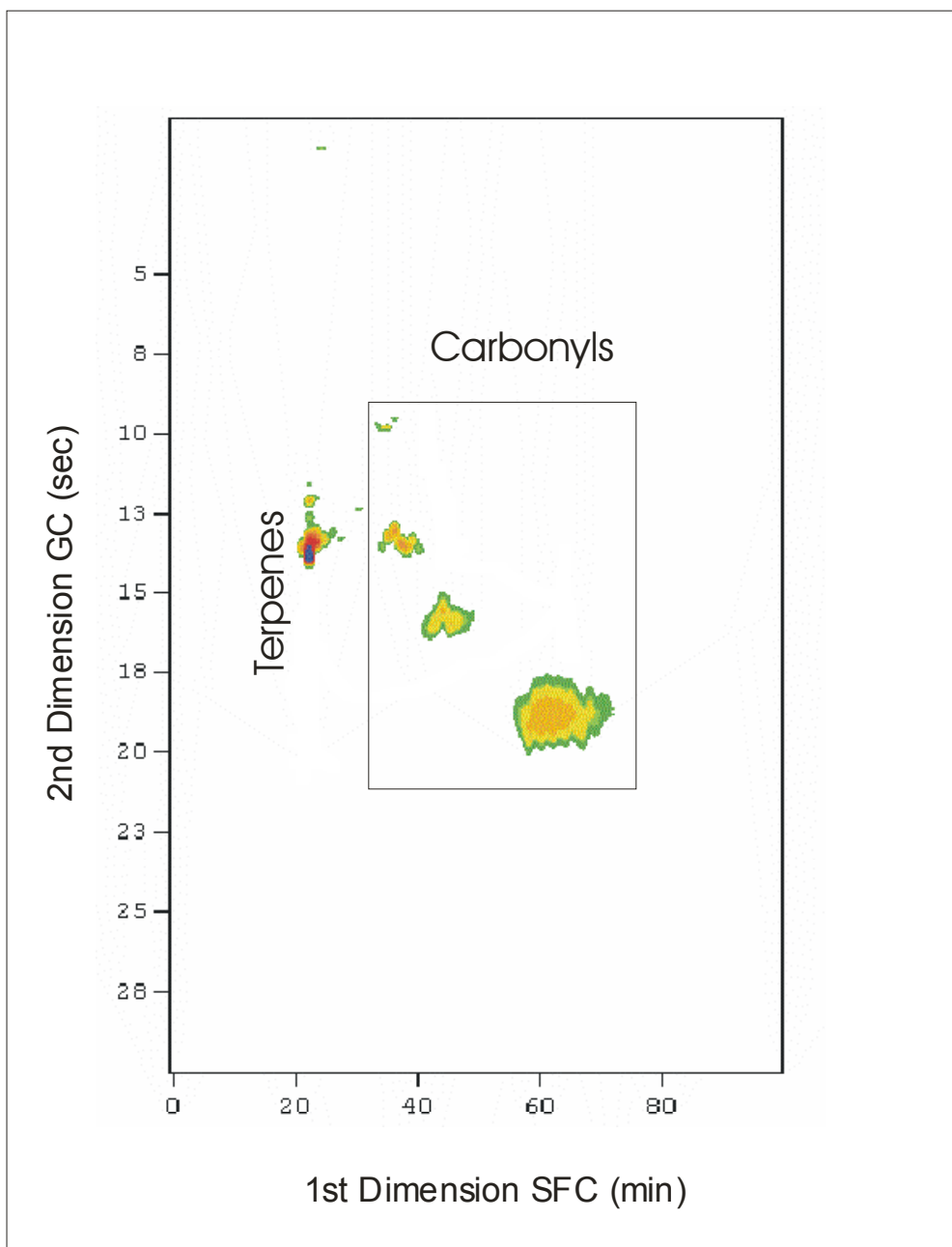
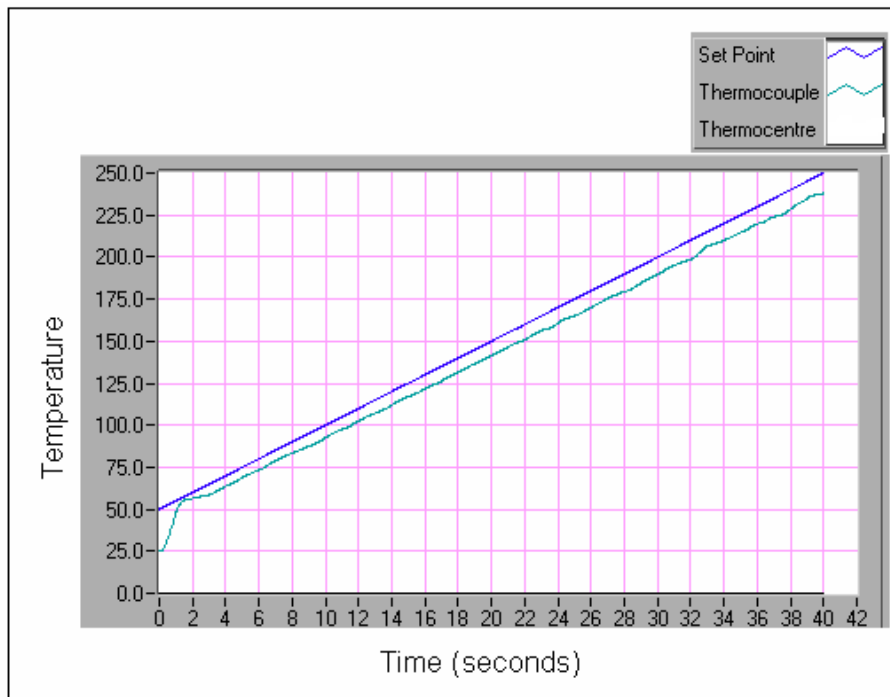
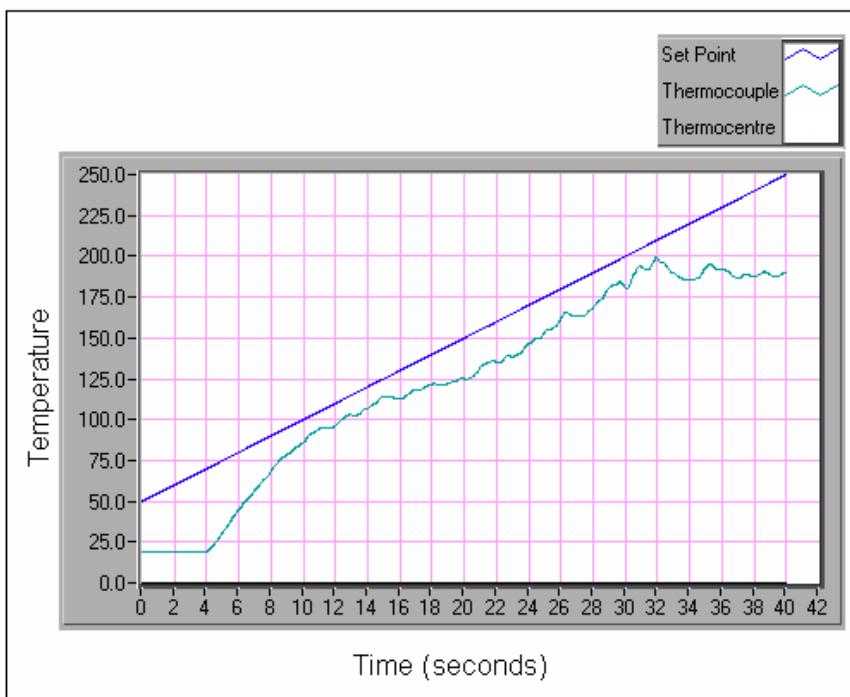


Figure 5.12 SFCxGC *Tagetes minuta* oil with a bad thermocouple contact on a 1 m stainless steel, GC capillary column.



**Figure 6.13.** Fast GC temperature ramp: good thermocouple contact with the column.



**Figure 6.14** Fast GC temperature ramp: bad thermocouple contact with the column.

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## 6.4 References

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# CHAPTER 7

## CONCLUSION

### 7.1 Supercritical fluid chromatography analysis

Chemical class separation with SFC produces very important information. With a silica-gel PLOT column, four essential oil samples (*Tagetes minuta*, *Artemisia afra*, *Cymbopogon citratus & flexuosus*, and *Pelargonium capitatum*) were separated into different chemical classes without the need to use either modifier or the backflush method. Terpene hydrocarbons are well separated from the oxygenated compounds. Oxygenates are partially separated into esters, ketones, aldehydes and alcohols.

The CO<sub>2</sub> mobile phase in SFC is compatible with the FID quantification of the separated essential oil groups and results about the relative amount of each eluted fraction were obtained. With SFC it is possible to separate samples at room temperature, near the critical temperature of the CO<sub>2</sub> mobile phase, to enhance the group separation. Operational conditions for SFC separation were obtained to separate the essential oils samples. It was found that near critical temperature (28 °C), 110 atm pressure and a flow rate of 480 ml/min (7.7 cm.s<sup>-1</sup>) and split of 50 ml/min to SFC-FID and 430 ml/min to fast GC gave fair group-type separation of essential oil samples especially for separating terpene hydrocarbons from the oxygenates. Volume flow rates apply to measurements of CO<sub>2</sub> flow after expansion to atmospheric pressure.

## 7.2 Comprehensive Two-dimensional SFCxGC analysis

SFC-FID on its own provides useful information about the relative percentages of some chemical classes contained in essential oil samples. To increase the amount of data that can be obtained from the sample mixtures, additional analysis of the SFC groups is required. Comprehensive coupling of SFC with fast temperature programmed gas chromatography by a flow modulator using a stopped-flow arrangement was used to separate four samples of essential oils. The separated peaks obtained from essential oils by SFC contained many components. Some of those co-eluting compounds are individually of interest to the flavour and fragrance industries. A measurement of individual components is often necessary. With fast temperature programmed GC, information regarding individual components contained in each class was obtained by effecting boiling point separation of the groups. The terpene hydrocarbons, separated from the oxygenates in SFC, is further separated into C10 monoterpenes, C15 sesquiterpenes and C20 diterpenes in some of the oils.

With favourable temperature conditions both in GC (temperature ramp) and SFC (near critical temperature of CO<sub>2</sub> mobile phase) different groups of esters were distinguished with SFCxGC. This feature was observed in a chromatogram of *Pelaragonium* (figure 6.5) where ester formates (C11), ester acetates (C12), ester propionates (C13), ester butyrates (C14) and tiglates (C14) were well differentiated. This demonstrates some of the SFCxGC instrument capabilities. SFCxGC shows an increased separation power over SFC one-dimensional separation of essential oils. This increased separation power is a result of the SFCxGC advantages which include the: increased peak capacity, sensitivity, and ordered separations based on chemical class.

By using the distinctive peak patterns observed in all four oils, it was possible to compare the oils and characteristic features were observed such as the presence of certain compounds in some of the oils and their absence in others. With the literature information on the composition of the oil and the use of standards it is possible to identify some of the major components in the oils. The possibility of obtaining the

three-dimensional chromatograms allows easy visualization of the oil components including the lower concentration peaks. The quality of the SFCxGC chromatogram was found to be dependent on the reproducibility of the fast GC that is influenced by the thermocouple placement on the column. GC reproducibility was found to be better than SFC reproducibility in consecutive SFCxGC runs. The SFC retention variability seems to be caused by (1) variation in the linear velocity of the mobile phase and (2) insufficient temperature control of the SFC column at the relatively low temperature (28 °C) by the GC oven.

The power of SFCxGC for fingerprinting of essential oils was illustrated by differentiating samples of *C. citratus* oil obtained from different geographical locations. Samples of *C. flexuosus* were also readily distinguishable from *C. citratus*.

Although SFCxGC is not faster than normal capillary GC, it should be appreciated that much more information can be obtained in the same time. A fingerprint pattern containing information on essential oils composition (especially chemical group composition) can be obtained. This information can be used for quality control purposes or identification of unknown essential oil sample mixtures by using pattern recognition. The opportunity to apply mass spectrometry to the second dimension separation would tremendously enhance the qualitative power of SFCxGC for compound identification. Without mass spectrometry, no identification of unknown components can be achieved, however, valuable information as to the chemical class and volatility of unknowns can be achieved. Fortunately, conventional MS scan speeds of 10 scans per second can effectively deal with the fast GC peaks of 0.5 sec width.

### 7.3 Possible future research work on SFCxGC system.

1. Additional work on representing a two-dimensional SFCxGC chromatogram is required that includes a bar polarity scale and integration of the Kovats Index scale to the fast GC retention axis.

2. The stability of the CO<sub>2</sub> pump flow and the SFC column temperature must be improved to alleviate the retention times shifts explained in the polarity separation.
3. Although the in-house built resistively heated fast GC shows acceptable retention time stability, this needs further attention to improve the appearance of the three dimensional peaks, also to allow automatic integration of these peaks for quantitative analysis. A reliable means of attaching the micro-thermocouple to the column or an alternative fast temperature probe is a prerequisite for improved GC retention time stability.
4. Mass spectrometry should be coupled in-line with the SFCxGC to alleviate the problem of peak allocation in known mixtures and to identify unknown components.

## APPENDIX A

Table A.1. Identified components of *Cymbopogon citratus* oil

No.	Components	Mass %
1	2,6 Dimethyloctane	0.1
2	<b>Myrecene</b>	<b>25.3</b>
3	Z- $\beta$ -Ocimene	1.0
4	E- $\beta$ -Ocimene	0.7
5	p-Cymene	0.5
6	trans-allo-Ocimene	0.1
7	Tetrahydrolinalool	0.3
8	Fenchone	0.2
9	Citronellal	0.3
10	$\beta$ -Patchoulene	0.2
11	Linalool	0.6
12	Camphor	0.1
13	<b>Neomenthol</b>	<b>3.3</b>
14	Terpinen-1-ol	0.4
15	<b>Linalyl acetate</b>	<b>2.3</b>
16	$\beta$ -Caryophyllene	0.3
17	<b>Neral</b>	<b>26.5</b>
18	Sabinol	0.1
19	<b>Geranial</b>	<b>33.7</b>
20	Nerol	0.8
21	Geraniol	1.9

Ref. E.A. Weiss, Essential Oil Crops, CAB International, 1997



**Table A.2.** Identified components of *Cymbopogon flexuosus* oil.

No.	Components	Mass %
1	$\alpha$ -Pinene	0.06-2.67
2	<b>Camphene</b>	<b>0.07-13.46</b>
3	$\beta$ -Pinene	0.1
4	Myrecene	1.93-4.33
5	$\alpha$ -Phellandrene	0.05-0.16
6	Limonene	0.035-3.03
7	$\beta$ -Phellandrene	0.11-0.40
8	(Z)- $\beta$ -Ocimene	0.05-0.20
9	<b>(E)-<math>\beta</math>-Ocimene</b>	<b>0.82-20.09</b>
10	<b><math>\gamma</math>-Terpinene</b>	<b>0.21-9.91</b>
11	Terpinolen	0.10-0.43
12	Citronellal	0.06-0.18
13	Linalool	0.77-9.95
14	<b>Neral</b>	<b>1.84-10.42</b>
15	$\alpha$ -Terpineol	0.06-1.42
16	Borneol	0.28-4.85
17	<b>Geranial</b>	<b>1.82-15.03</b>
18	Geranyl acetate	0.62-7.74
19	Citronellol	0.23-1.24
20	Nerol	0.14-0.32
21	<b>Geraniol</b>	<b>3.0-74.72</b>

Ref. E. Chishowa, D.R. Hall, D.I. Farman, Flavour and Frag. J., 13 (1998) 29-30

**Table A.3.** Identified components of *Tagetes minuta* oil.

No.	Components	%
1	$\alpha$ -Pinene	0.06
2	Ethyl-2-methylbutyrate	0.08
3	Sabinene	0.96
4	Myrecene	0.1
5	$\alpha$ -Phellandrene	0.09
6	$\alpha$ -Terpinene	0.02
7	Limonene	7.24
8	$\beta$ -Phellandrene	0.07
9	(E)-2-hexanal	0.06
<b>10</b>	<b>(Z)-<math>\beta</math>-Ocimene</b>	<b>28.49</b>
11	$\gamma$ -Terpinene	0.05
12	(E)- $\beta$ -Ocimene	0.39
<b>13</b>	<b>Dihydrotagetone</b>	<b>30.3</b>
14	allo-ocimene	0.32
15	(Z)-Tagetone	0.25
16	Decanal	0.12
17	(E)-Tagetone	4.8
18	$\beta$ -Caryophyllene	0.47
19	(Z)-Tagetonone	1.87
<b>20</b>	<b>(E)-Tagetonone</b>	<b>15.35</b>
21	Bicyclogermacrene	0.1
22	isopiperitenone	0.26

Ref. J. Chalchat, R.P. Granny, A. Muhayimana, J. Essent. Oil Res. 7 (1995) 375-386

**Table A.4.** Identified components of *Artemisia afra* oil.

No.	Components	Mass %
1	Tricyclene	0.1-0.2
2	$\alpha$ -Pinene + $\alpha$ -Thujone	0.4-1.1
3	$\alpha$ -Fenchene	0.1-1.0
4	<b>Camphene</b>	<b>0.3-3.9</b>
5	$\beta$ -Pinene	0.1-0.7
6	Sabinene	0.1-1.1
7	Myrcene	0.1-1.1
8	$\alpha$ -Terpinene	0.1-1.1
9	Dehydro-1,8-cineol	0.1-0.2
10	Limonene	0.1-0.5
11	<b>1,8-Cineol</b>	<b>0.1-27.9</b>
12	( <i>E</i> )- $\beta$ -Ocimene	0.1-0.3
13	$\gamma$ -Terpinene	0.3-1.9
14	p-Cymene	0.3-2.0
15	Terpinolene	0.1-0.5
16	<b>Artemisia ketone</b>	<b>6.3-41.9</b>
17	<b>Santolina alcohol</b>	<b>3.1-10.1</b>
18	$\alpha$ -Thujone	1.0-2.9
19	Artemisyl acetate	0.1
20	$\beta$ -Thujone	Trace
21	cis-Sabinene hydrate	0.2-0.5
22	Artemisia alcohol	0.3
23	<b><math>\alpha</math>-Caopaene</b>	<b>8.5-27.1</b>
24	trans-Sabinene hydrate	1.8-4.4
25	cis-p-menth-2-en-1-ol	0.2-0.4
26	Bornyl acetate	0.3-1.5
27	$\beta$ -Caryophyllene	0.5-2.3
28	Terpinen-4-ol	0.1
29	Myrtenal	0.1
30	Trans-p-meth-2-en-1-ol	0.2-0.3
31	$\delta$ -Terpineol	0.1-2.5
32	<b>Borneol</b>	<b>0.6-3.4</b>

Ref. E.A. L.S. Chagonda, C. Makanda, J-Claude Chalchat, Flavour and Frag. J., 14 (1999) 140-142.

**Table A.4.** Identified constituents of *Artemisia afra* oil.

33	$\alpha$ -Terpineol	0.1-2.5
34	Bicyclogermacrene	0.2-0.5
35	Piperitol	0.1-0.7
36	$\delta$ -Cadinene	0.5.-0.8
37	Cuminaldehyde	0.5
38	Myrtenol	0.1
39	Calamenene	0.1-0.9
40	Cis-Carveol	0.1
41	trans-Caryophyllene oxide	0.1
42	Methyl linolenate	0.1
43	Germacene-D-4-ol	0.1
44	P-Cymen-8-ol	0.1
45	Spathulenol	0.1
46	T-muurolol	0.5
47	Intermomedeol	0.4

Ref: L.S. Chagonda, C. Makanda, J-Claude Chalchat, Flavour and Frag. J., 14 (1999) 140-142.

**Table A.4.** Identified constituents of *Pelargonium capitatum X radens* oil.

no.	Components	%
1	$\alpha$ -Pinene	1.00
2	Myrecene	0.30
3	<i>cis</i> - $\beta$ -Ocimene	0.30
4	<i>cis</i> -Rose oxide	0.20
5	<i>trans</i> -Rose oxide	Ng
6	<b>Linalool</b>	<b>4.60</b>
7	Menthone	0.40
8	<b>Isomenthone</b>	<b>7.80</b>
9	$\alpha$ -terpineol	0.30
10	<b>Citronellol</b>	<b>19.00</b>
11	<b>Geraniol</b>	<b>21.50</b>
12	Geranial	Ng
13	<i>trans</i> -Citral	1.10
14	<b>Citronellyl formate</b>	<b>8.50</b>
15	<b>Geranyl formate</b>	<b>9.50</b>
16	Geranyl acetate	0.50
17	$\beta$ -Bourbonene	0.70
18	$\beta$ -Caryophyllene	0.80
19	Citronellyl propionate	0.20
20	<b>Guaiadiene 6,9</b>	<b>7.20</b>
21	Geranyl propionate	1.60
22	Germacrene D	2.30
23	Citronellyl butyrate	1.00
24	Geranyl butyrate	1.20
25	Phenylethyl tilgate	0.70
26	Citronellyl tilgate	0.10
27	Geranyl tilgate	1.30
28	Geranyl tilgate	1.30

Ref. E.A. Weiss, Essential Oil Crops, CAB International, 1997

**Table A.6** Reproducibility results of SFCxGC run-to-run of *C. citratus* oil for second dimension retention.

Peak no.	2tr1	2tr2	2tr3	2tr4	Mean	STD	%RSD
1	14.91	14.95	14.90	14.89	14.91	0.02	0.15
2	15.20	15.85	15.75	15.57	15.59	0.25	1.59
3	16.40	16.50	16.70	16.46	16.52	0.11	0.68
5	23.03	22.85	22.75	23.07	22.93	0.13	0.57
6	21.14	20.10	19.55	21.04	20.46	0.66	3.24
7	18.25	17.91	17.85	17.93	17.99	0.16	0.78
9	18.00	16.80	16.25	17.93	17.25	0.75	4.31
8	15.80	14.60	14.10	15.07	14.89	0.63	4.21
						<b>0.34</b>	<b>1.94</b>

**Table A.7** Reproducibility results of SFCxGC run-to-run of *C. citratus* oil for first dimension retention

Peak no.	1tr1	1tr2	1tr3	1tr4	Mean	STD	%RSD
1	22.10	18.50	19.50	19.97	20.20	1.31	6.57
2	22.50	19.00	20.00	21.05	20.64	1.30	6.28
3	21.90	18.50	20.10	21.03	20.38	1.26	6.18
5	44.50	39.50	42.25	43.45	42.43	1.87	4.40
6	49.00	40.50	43.50	45.30	44.58	3.08	6.90
7	40.50	33.50	34.50	35.09	35.90	2.72	7.57
9	72.00	61.50	65.50	67.26	66.57	3.77	5.66
8	66.50	57.00	59.50	61.49	61.49	3.49	5.71
						<b>2.35</b>	<b>6.16</b>

APPENDIX B

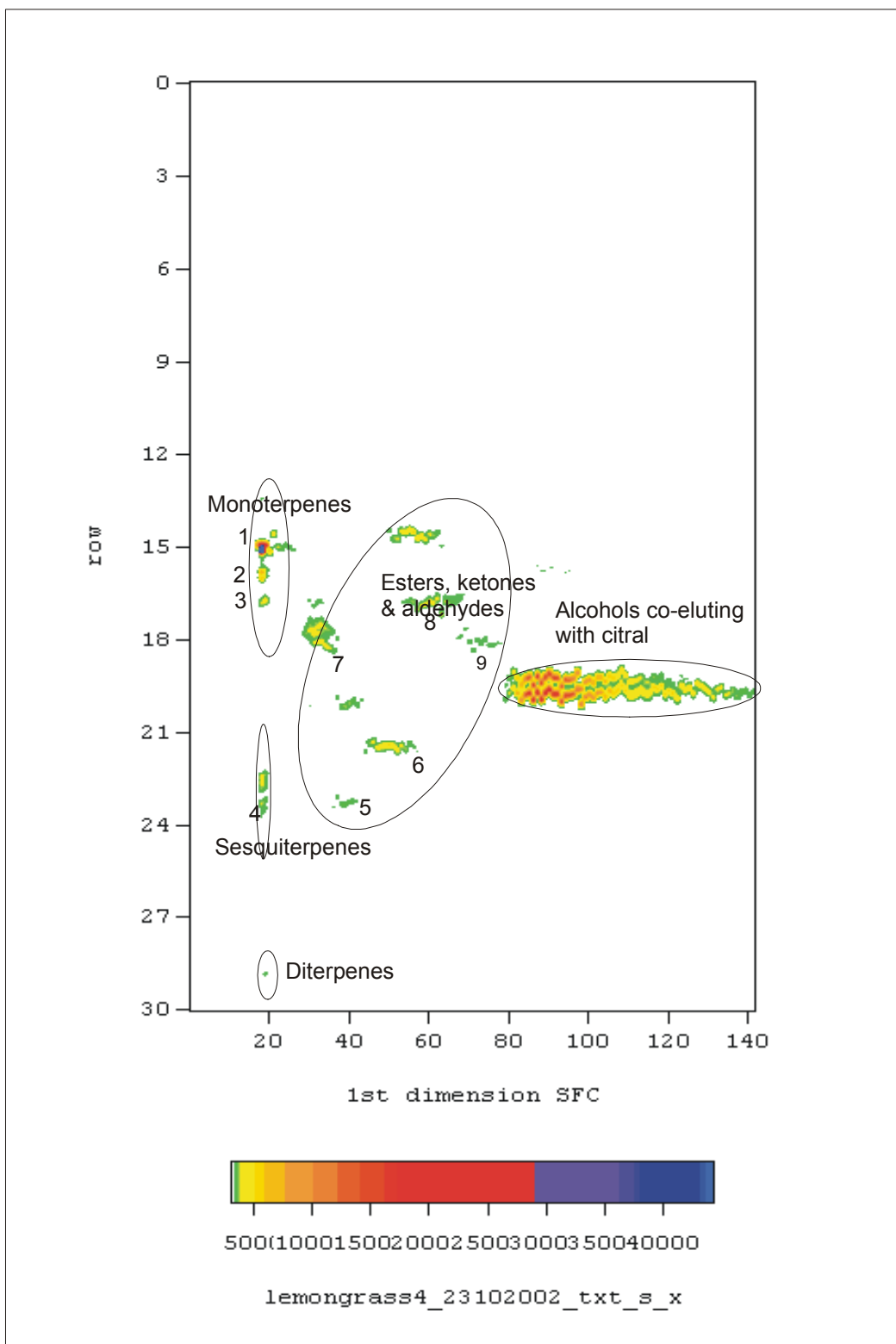
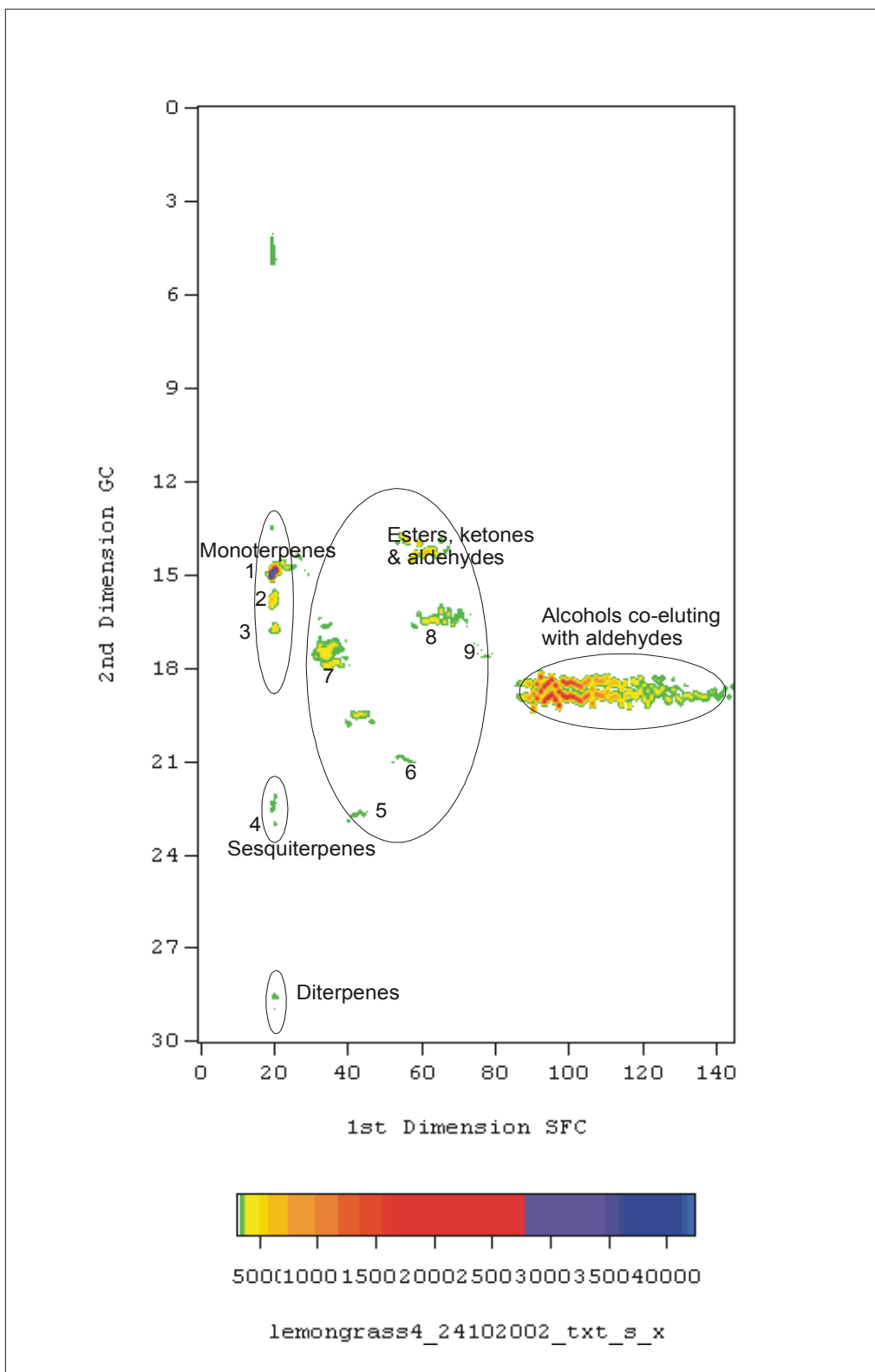
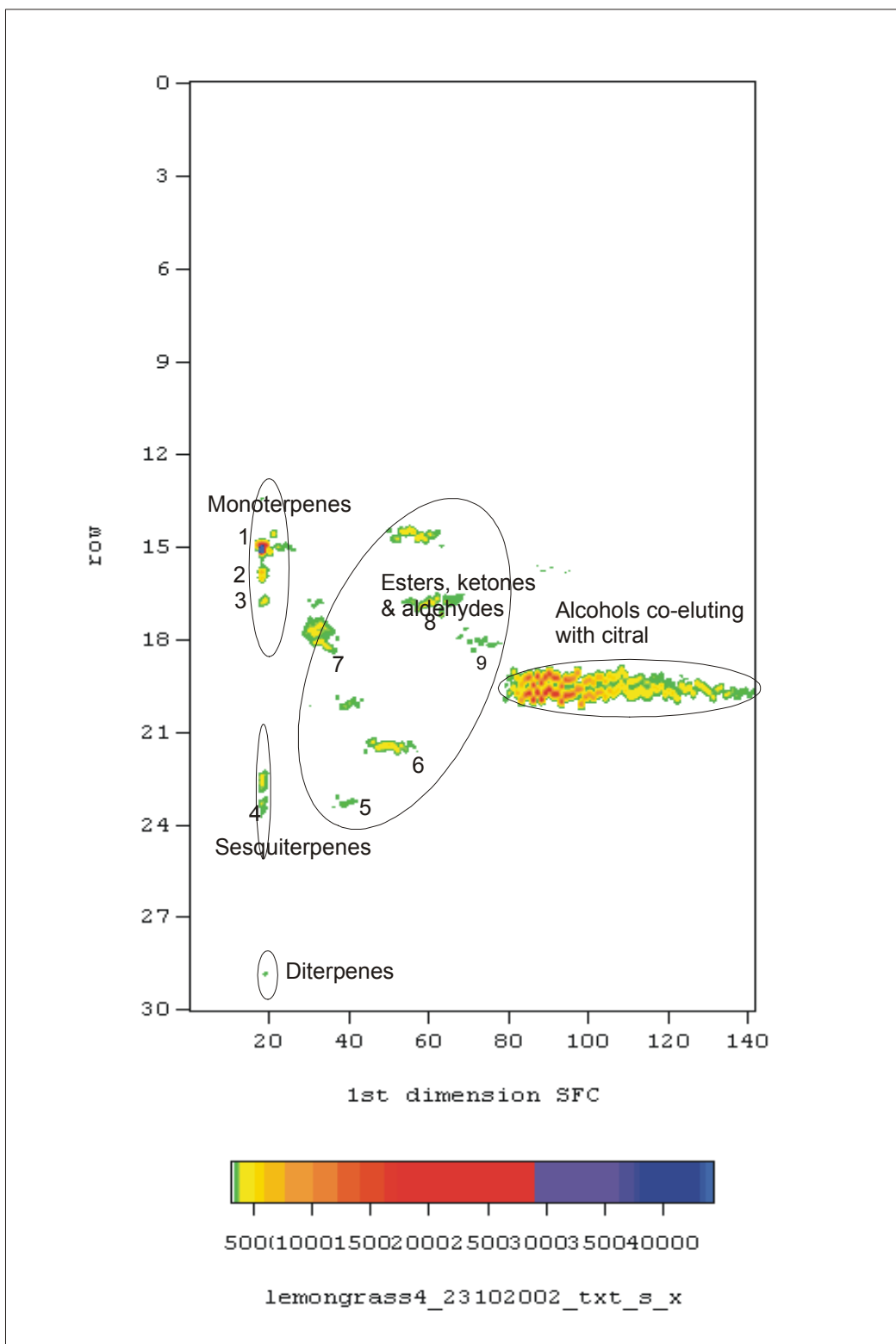


Figure B.1 SFCxGC chromatogram of *Cymbopogon citratus* sample 4 (run 1)

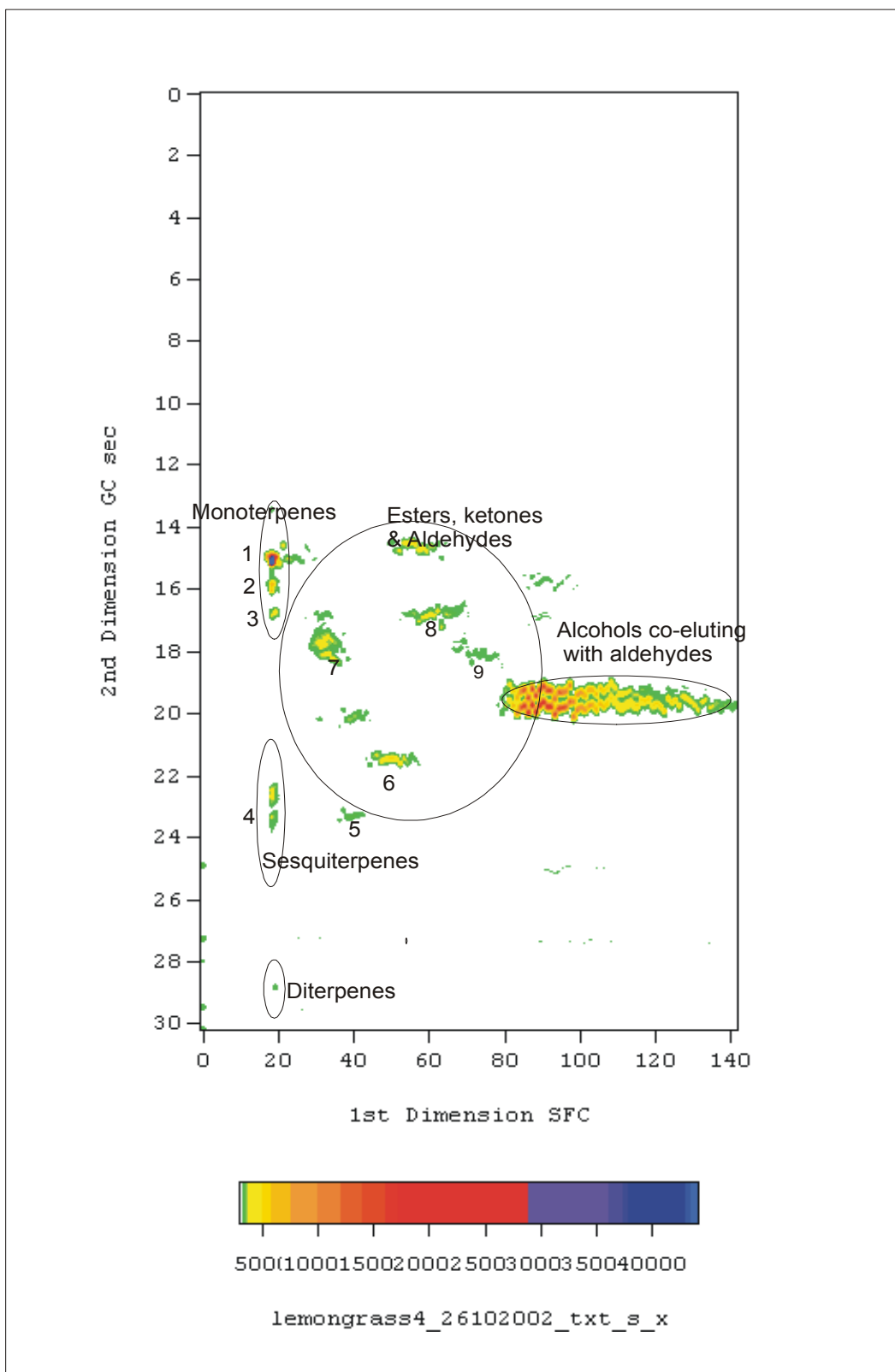


**Figure B.2** SFCxGC chromatogram of *Cymbopogon citratus* sample 4 (run 2)





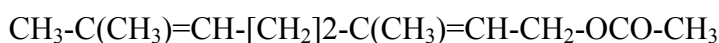
**Figure B.3** SFCxGC chromatogram of *Cymbopogon citratus* sample 4 (run 3)



**Figure B.4** SFCxGC chromatogram of *Cymbopogon citratus* sample 4 (run 4)

**APPENDIX C****Some of essential oil components chemical structures**

1. Geranyl acetate : (E)-3,7-dimethyl-2,6-octadien-1-yl ethanoate



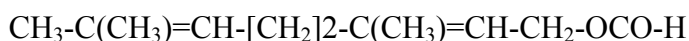
2. Citronellyl propionate : 3,7-dimethyl-6-octen-1-yl propanoate



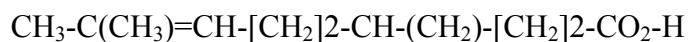
3. Geranyl propionate : (E)-3,7-dimethyl-2,6-octadien-1-yl propanoate



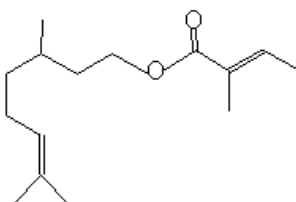
4. Geranyl formate : (E)-3,7-dimethyl-2,6-octadien-1-yl methanoate



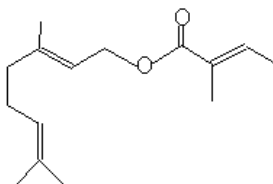
5. Citronellyl formate : 3,7-dimethyl-6-octen-1-yl methanoate



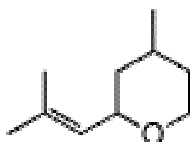
6. Citronellyl tiglate : 2,6 Dimethyl Octenyl Tiglate



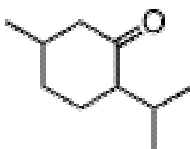
7. Geranyl tiglate : 3,7, Dimethyl-2,6-Octadien-1-yl-Tiglate



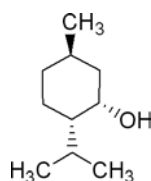
8. (-)-cis-rose oxide: tetrahydro-4-methyl-2-(2-methyl-1-propenyl)-2,5-cis-2H-pyran,



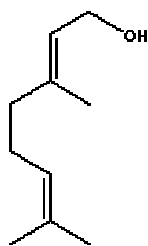
9. Menthone : 5-Methyl-2-(1-methylethyl)cyclohexanone



10. Neomenthol :



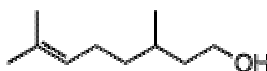
11. Geraniol :  $(\text{CH}_3)_2\text{CCH}(\text{CH}_2)_2\text{C}(\text{CH}_3)\text{CHCH}_2\text{OH}$



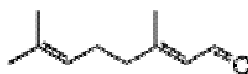
12. linalool : 3,7-dimethylocta-1,6-dien-3-ol, 2,6-dimethylocta-2,7-dien-6-ol (R, S, and racemate)



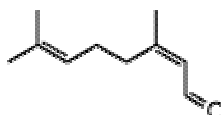
13. citronellol : 3,7-dimethyl-6-octen-1-ol,  $\beta$ -citronellol, (+)-citronellol, rodinol, DL-citronellol



14. geranial : (E)-3,7-dimethyl-2,6-octadienal

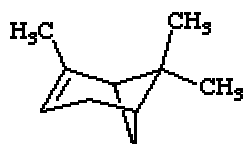


15. neral : 3,7-dimethyl-(Z)-2,6-octadienal



16. (-)-a-Pinene : (1S,5S)-2,6,6-trimethylbicyclo[3.1.1]hept-2-ene

(-)-b-Pinene : (1S,5S)-6,6-dimethyl-2-methylenebicyclo[3.1.1]heptane



$\alpha$ -pinene



$\beta$ -pinene

17. Camphene : 2,2-dimethyl-3-methylene-bicyclo[2.2.1]heptane; 2,2-dimethyl-3-methylene norborane



