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[1]. 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[2]:

- 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[4]. 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) [5], Headspace-GC[6] 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[7] and further improvement by Martin[8], 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[9]. 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[6], SPME[5], and direct thermal desorption[10]. 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[11].

Headspace analysis is a powerful tool for solving problems such as trace detection, or for checking a wide variety of different materials[12]. 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[11].

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[13]. 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[14]. 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[14]. For each fiber tested, the sampling time is optimized for reproducible results. The 7-µm-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[15]. 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[16] 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[16]

Description	Heating rate (°C/min)	Column length (m)	Column i.d. (μm)	Analysis time (min)	Peak Width (s)
Ultra Fast	60 - 1200	5 – 2	100 - 50	~ 1	0.2 – 0.05
Fast	20 - 60	15 – 5	250 - 100	~ 10	5 - 0.5
Conventional	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[17]. 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[18]. 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[19]. 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[18], 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[16] :

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[19]. Ramp rates have increased from 1 – 2 ^{o}C/sec to 150 ^{o}C/sec[19]. 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[20] in 1958 and further reaffirmed by Rounbehler in 1991[21]. Numerous resistively heated systems have recently been developed to allow faster temperature programming and cooling times. They can achieve heating rates of 20 ^{o}C/sec (1200 ^{o}C/min) and cool down very rapidly from 300 ^{o}C to 50 ^{o}C in less than 1 minute[16].

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[22]. 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[23]. A fast electrometer board has been developed for the flame ionization detector[22] to increase the detection response speed of the FID.

Hail and Yost[24] used resistively heated aluminium clad capillary columns. Overton and co-workers[25] 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 ^{o}C.

Other researchers also heated aluminium-coated fused-silica columns directly by passing a current through the metallic outer layer[26,27]. 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[24.] 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[28]. 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[29]. 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[29].

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[30]. 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[31] 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:

$$n_c = (1 + \frac{N^{1/2}}{r}) \ln(1 + K_i^{'}) \qquad \textbf{[3-1]}$$

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[32, 33].

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[30]. 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[34].

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[30] 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[35]. 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.

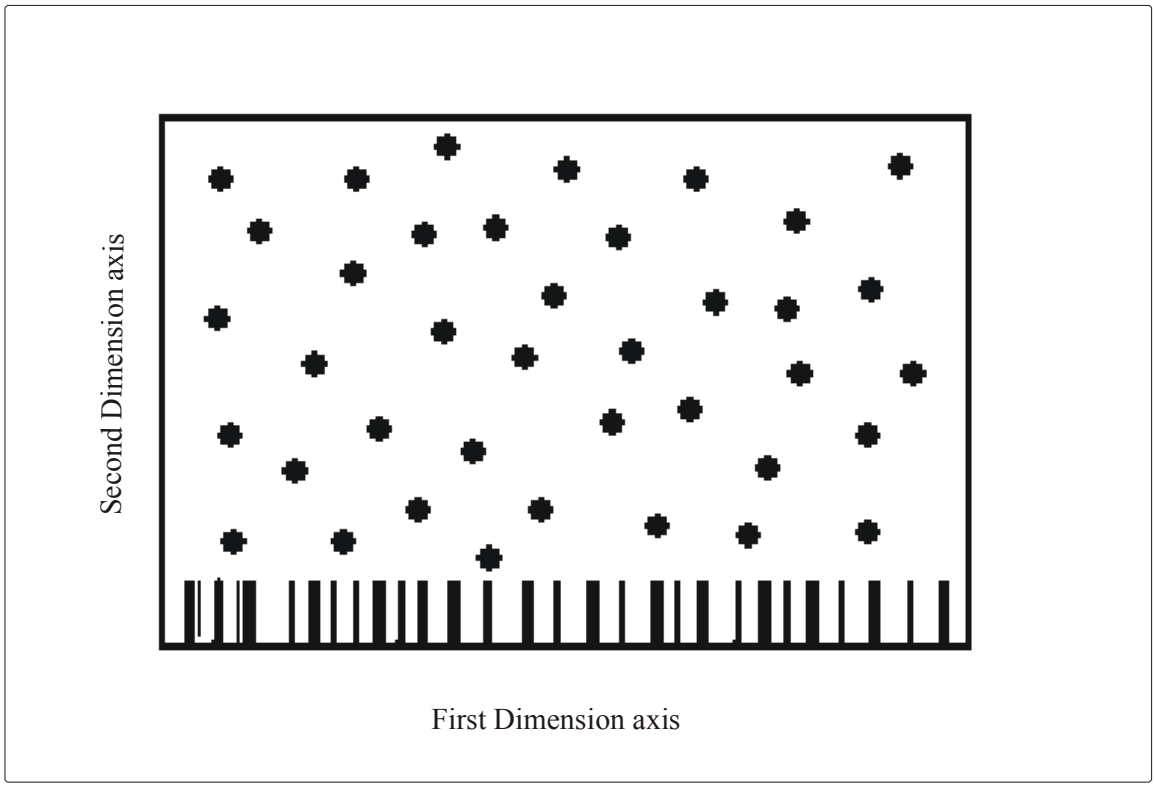

Figure 3.1. A two-dimensional plane representation of a complex sample, separated using two independent separation methods[35] (depicted by the stars) and a representation of the same sample separated using only one separation axis (Represented by the lines).

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[36]. 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[37].

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[38]. It has been shown that peak capacity is in practice less than the product of the two dimensions of comprehensive-multidimensional chromatography[39]. Also that an order of magnitude improvement in peak capacity is expected from a GCxGC analysis as compared to one-dimensional GC[40] .

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[41]. 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:

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

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 :

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

where α 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 [33,41]. 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[42].

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[35], 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[35]. 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 $n=S''$[35]. 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[35].

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[42]. 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[30]. 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[45]. 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[41]. 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[30]. 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[43]. 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[44].

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[30]. 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.

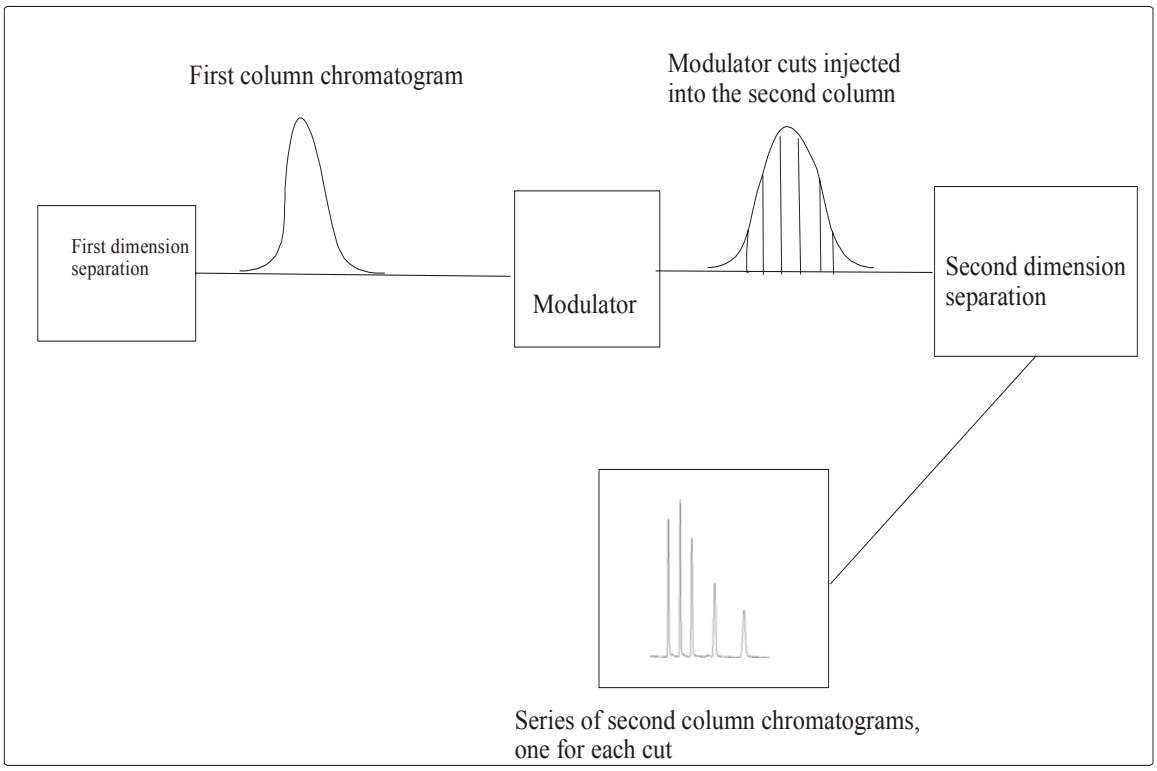

Figure 3.2 Representation of the steps involved in comprehensive two-dimensional chromatography.

3.3.3.1 LCxLC

Two-Dimensional liquid chromatography was first demonstrated by Erni and Frei[37]. 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[45] 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[45].

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[46] in the early 1980s. Though the system possessed some features of comprehensive multidimensional chromatography, more development of the system was needed. Liu and Phillips[47], 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[47]. 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[48,49,50] . 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[51]. 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^{o}C[52]. 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 bandwiths.

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[53] 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_2 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[54]. 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.

3.3.34 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[42]. 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[47], where volatility separation in the first dimension normally precedes polar separation in the second dimension.

This SFCxGC is different to the previously demonstrated SFCxGC[53] instrumentation, where a standard GCxGC[47] 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[42]. 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[55]. 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[54].

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[42]. 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[42]. 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[56]. 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[57]. 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[58].

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)[59]. 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[58].

Recently, better separations of essential oils were reported with GC-TOF-MS[59,60]. 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[58].

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 1st 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[30]. 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 of MDGC applications to specific components for fragrance analysis, such as analysis of enantiomeric constituents of cold-pressed and distilled fruits, have been reported [61,62].

The recently described technique of comprehensive two-dimensional gas chromatography (GCxGC)[47] 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[63] 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[64] 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[65].

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[66]. 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[67]. 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[68] , the analysis of a wide range of oils[69] and also for chiral analysis[70]. 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_2 mobile phase that is compatible with the universal flame ionization GC detector. Yamauchi and Saito[71] 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[72]. 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_2. 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.

3.6 References

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