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3 MULTIDIMENSIONAL CHROMATOGRAPHIC TECHNIQUES AND THE ANALYSIS OF ESSENTIAL OILS

3.1 Multidimensional Chromatographic Techniques

A Multidimensional chromatographic technique is a technique that employs two or more methods of separation in series to effect the separation of components, which are otherwise difficult to achieve using one-dimensional systems. According to J.C. Giddings [1], a multidimensional separation is characterised by two conditions. Firstly, a sample is analysed by two or more analysis steps of different characteristics and secondly, components separated in the previous step generally remain separated until the end of the analysis process. Because of these two conditions, multiple parameters (elution times, fragmentation pattern, etc) are required to distinctively describe the final position of a component after a multidimensional separation. Therefore two components will be successfully separated and have different final positions, if at least they have a difference in one of the separation parameters [1].

The first attempt at creating a multidimensional chromatographic separation was performed along the axes of a paper sheet (TLC×TLC) in 1944. This was followed by gas chromatography coupled to electrophoresis in 1948 and a two-dimensional electrophoresis (electrophoresis-electrophoresis) system in 1951 [2]. In 1968 Deans demonstrated the possibility of heart-cutting two-dimensional gas chromatography *via* the *Deans switch* valve to transfer part of a GC chromatogram into a second column with different polarity [3]. This was followed by the coupling of isoelectric focusing with electrophoresis (1975-1982) to achieve a two dimensional system for the analysis of proteins [2]. In 1980 Majors also reported the coupling of liquid chromatography to capillary gas chromatography [3].

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The early 1990's witnessed the development of new techniques in multidimensional column chromatography and comprehensive two-dimensional column chromatographic techniques were born. In 1990 Bushey and Jorgenson described the first comprehensive 'all-inclusive' multidimensional column chromatography. They coupled an ion exchange first dimension column with a size exclusion liquid chromatographic second dimension column. The two dimensions were interfaced by means of two sample loops mounted on an eight–port valve to effect the subsequent separation of all injected solutes in the serially coupled columns [4]. In 1991 Phillips and Liu [3, 5] broke new ground for a comprehensive two-dimensional gas chromatographic (GC×GC) system. In this technique the authors utilised a thermal desorption modulator to trap and focus sample components eluting from the first dimension column before being introduced into the second dimension column as sharp solute pulses [5].

The drive for the use of multidimensional techniques has its roots in the inability of linear systems to handle complex samples. One-dimensional techniques lack the power of clearly and efficiently separating complex samples, *i.e.*, they have insufficient peak capacities to adequately resolve complex samples and they end-up giving overlapping component peaks. Peak capacity is the maximum number of peaks that can be separated by a given separation mechanism with satisfactory resolution between neighbouring components [1]. To efficiently separate a certain number of components in a sample a peak capacity (n_c) of greater or equal to the number of components (m) in the sample is required. The use of multidimensional systems greatly enhances the n_c from that of a single column, to be between the sum ($n_{c1} + n_{c2} \ldots + n_{cn}$) and the product ($n_{c1} \times n_{c2} \ldots \times n_{cn}$) of the number of columns or techniques serially coupled [1, 2, 6]. The peak capacity is related to the theoretical plate number N [1], which signifies the column efficiency by the following equation:

$$n_c = \theta \sqrt{N} \approx \frac{1}{2} \sqrt{N} \tag{3.1}$$

The constant θ, which depends on the retention time range, is approximately 0.5 [1].

According to the above equation increasing n_c by a factor of two requires quadrupling the theoretical plate number of the column. In a one-dimensional system this implies increasing

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the column length four times, which substantially increases the analysis time, respective cost and the technical difficulty of working with such columns. On the other hand, the theoretical plate number and n_c can be improved more easily without substantially increasing the column length by using multidimensional techniques and exploiting the multiplicative increase in n_c they create [1, 6].

The amenability of analytical samples to a separation or an analysis system does not entirely depend on sample complexity, *i.e.*, the number of components (m) in a given sample. There is another factor that affects the effective separation of samples by a certain analysis system called *sample dimensionality* or *sample variability*. Giddings defined sample dimensionality as: the number of independent variables that must be specified to identify the components of a sample. This parameter measures the make-up of a sample in terms of the differences that exist between components and the parameters required to separate them from each other [7]. For example, if a sample entirely consists of straight chain-hydrocarbons it has one dimension, boiling point difference. Therefore, it is theoretically possible to fully separate such components using a one-dimensional separation system with high enough n_c utilising a volatility-based separation. The use of a multidimensional system to separate such samples gives no actual increase in separation power, as there will be strong correlation between the successive parts of the system(s) used [7].

On the other hand, if a sample contains compounds from a number of homologous series, such as hydrocarbons, alcohols, esters or aldehydes, the sample dimensionality or variability is not single and simple any longer. The use of only volatility based separation will not effect good separation as there will be overlapping of components of similar boiling points. Aromatic hydrocarbons with almost the same boiling points as non-aromatic compounds can serve as an example. The separation of such samples requires a system which is capable of using more than one variable to effect separation. The use of a multidimensional separation method with higher n_c and extra separation parameters is not only useful but a necessity. Consequently, the success of multidimensional techniques in the analysis of complex samples in terms of defining variables needed to identify them, depends entirely on the availability and effectiveness of the required parameters in the system utilised to separate and

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identify the individual components *viz*. boiling point, polarity, size, etc. [7]. The analysis of multivariable samples using multidimensional systems not only is effective in terms of its separation power but it also provides very ordered chromatograms which have the potential advantage of being much more interpretable. In ordered chromatograms, patterns of peaks define members of a homologous series (aliphatic, mono-aromatic, di-aromatic, etc) and help to recognise chemical classes easily.

3.1.1 Peak Capacity and the Statistical Component Overlap Theory

To separate a number, m, of components in a certain sample with a unit resolution between two adjacent peaks, one needs a system with at least equal or more peak capacity, n_c. As n_c represents the maximum attainable number of resolved peaks in a chromatogram, the above statement is theoretically correct. However, this is only true if the component peaks of a sample are evenly spaced during the separation process. In reality, that is far from the case. In most complex samples peaks are randomly spaced and sometimes overlap with each other even when the separation space has much greater n_c than theoretically needed for complete resolution of the analytes at hand. This overlap of component peaks greatly reduces the amount of sample components that can be successfully resolved by a system of given n_c [1].

To explain and understand the component overlap theory it is necessary to look into some of the variables that explain the make-up of a chromatographic plot. The number of visible peaks (p) of a sample of m components that can be resolved by a given n_c ($m = n_c$) is given by equation (3.2) [1, 8]:

$$p \approx me^{-\frac{m}{n_c}} \approx 37\% \qquad (3.2)$$

From equation (3.2) it is clear that a system with peak capacity of n_c is only able to resolve less than 50% (≈ 37%) of the number of sample components presented to it due to the extensive overlap of peaks during separation. Furthermore, the numbers of single component peaks (s) that can be expected are much less than the number of p, as can be referred from equation (3.3) [1, 8].

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$$s \approx me^{-\frac{2m}{n_c}} \approx 18\% \tag{3.3}$$

Although the use of linear systems with n_c in excess of the number of components in the sample ($n_c >> m$) could address this problem, the attempt to design or use such systems has its own complications in terms of length of analysis time and excessive cost. The true remedy, as already suggested above, comes from the use of multidimensional methods, which have n_c values much higher compared to any linear chromatographic techniques [1].

The n_c of a truly orthogonal comprehensive two-dimensional chromatographic system, with uncorrelated separations and complete transfer of sample components between the two chromatographic dimensions without loss of resolution, is a product of the n_c of the individual columns [5, 9, 10]. This enhanced n_c makes a comprehensive two-dimensional chromatographic system more powerful to resolve complex mixtures than its one-dimensional counterpart. However, the multiplicative rule is only an estimation of the actual n_c that can be theoretically reached in two-dimensional separations. Correlations of solute retentions, *i.e.* lack of complete orthogonality between the two-dimensions reduce the available separation space to a restricted region [10]. If there is too much communication or cross-information between the two dimensions much of the separation space is unoccupied or even completely inaccessible and sample components tend to cluster along a diagonal. Reducing cross-information between successive columns increases the efficiency of information generation from multidimensional separations [1].

Mathematically, the n_c of an orthogonal two-dimensional separation system is given by equation (3.4) [1, 6]:

$$\boldsymbol{n}_{cT} \approx \boldsymbol{n}_{c1} \times \boldsymbol{n}_{c2} \tag{3.4}$$

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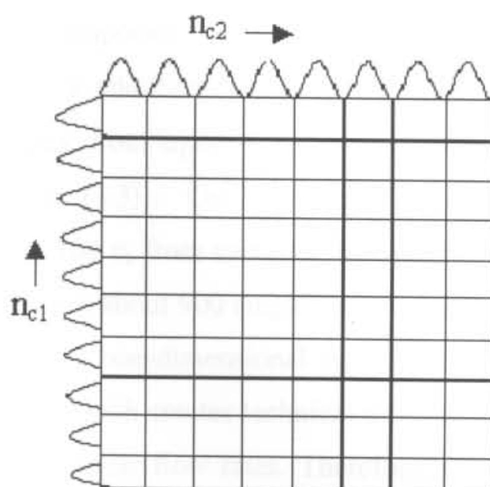

Figure 3.1 Orthogonal n_c of a 2D chromatograph, square boxes represent resolution units in the 2D space. The area of the plane represents the total theoretical peak capacity (n_{cT}) of a perfectly orthogonal system. But the available n_c is determined by the retention correlations between the two dimensions, which may be reduced to a one-dimension peak capacity if the two systems are highly correlated *i.e.*, with the same separation mode in the two dimensions. Therefore, the actual available n_c is usually smaller than the theoretical n_c [6, 10].

The use of two-dimensional chromatography does not directly reduce peak overlap. In the true sense, peak overlap is even worse in two-dimensional systems due to the overlap of peaks occurring in both the first and second dimensions. As a result the amount of p that can be expected is further reduced to about 16% and the number of s to about 9% as can be inferred from equations (3.5) & (3.6) [8]:

$$p \approx me^{-\frac{2m}{n_c}} \approx 16\% \tag{3.5}$$

$$s \approx me^{-\frac{4m}{n_c}} \approx 9\% \tag{3.6}$$

Fortunately, the enormous n_c and extra space that can be obtained from multidimensional systems substantially reduces peak saturation, given by the ratio of the number of components divided by n_c, m/n_c. And the resulting statistical-overlap of component peaks from complex samples is reduced as component peaks are allowed to spread out across additional coordinates [7]. This large n_c gives multidimensional techniques the upper hand in

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resolving large numbers of components that cannot be practically achieved by a single column system. For example, a one-dimensional chromatographic system with n_c of 1000 (with 4×10^6 theoretical plates; from equation (3.1)) can resolve approximately 180 single-component peaks (equation (3.3)). On the other hand, a two dimensional gas chromatographic system with 100 n_c from each column will have an n_{cT} of 10,000 (equation (3.4)) and can effectively resolve about 900 single-component peaks (equation (3.6)) [8]. To produce the same results from a one-dimensional system calls for the use of an extremely long and narrow bore column, which creates technical difficulties of working with very high pressures and high carrier gas linear flow rates. Therefore, the use of comprehensive two-dimensional systems for the analysis of complex samples does not only provide high separation power but also relieves one of the technical difficulties of working with one-dimensional systems [1].

3.2 Comprehensive Two-Dimensional Gas Chromatography (GC×GC)

Comprehensive two-dimensional gas chromatography (GC×GC) is a multidimensional technique in which two capillary gas chromatographic columns of different selectivity are serially coupled to provide better and efficient chromatographic resolution. In its basic form, all sample components eluting from the first column are completely trapped and focused by a modulating interface to be re-injected in the form of distinctive pulses into the second dimension column. The second dimension column with different separating characteristics, shorter length and narrower bore than that of the first column further analyses and separates co-eluting first dimension solutes to give sharp peaks [5, 9].

In GC×GC the second dimension column must be operated fast enough to preserve the information contained in the first dimension separation step [9, 11]. Should a second dimension column be too slow the information obtained from the first column will be degraded, as there will be not sufficient number of cuts across each first dimension peak to preserve its shape. Peaks already separated in the first dimension will overlap. Also, if the second dimension column is too fast relative to the first dimension column, poor use of the analysis time results in lower second dimension resolutions [11, 12].

Solutes injected into the second dimension column at a particular time are rather few and of similar volatility and separation is a function of chemical class rather than vapour pressure differences. For this reason the second dimension column does not need large n_c. Although the total analysis in GC×GC is usually a temperature programmed run, the individual second dimension sub-sample injections are so short in time that they can in practice be considered as isothermal runs ($\Delta T \sim 0.1^{o}C$/chromatogram) [12].

Overall, during a GC×GC run there must be no loss of information obtained from the first dimension during transfer to the second dimension column or in the process of solute separation in the second dimension column [12]. A complete set of secondary chromatograms is then generated in real time as the primary chromatogram develops. This is only possible if the two dimensional system is in an orthogonal arrangement in which the mode of separation in the first dimension column is independent of the analytical separation in the second dimension [9, 13].

The success and orthogonality of a GC×GC system depends on the proper choice of the two columns, the interfacing modulator and the proper operation of their combination. The type of sample analysed may also give a false signal about the orthogonality and efficiency of the system. A sample containing components of similar properties, for example, only *n*-alkanes, may not show the true orthogonality of a GC×GC system. To test the orthogonality of a system the test sample itself must contain substances distributed over the whole range of properties relevant to the first and second dimension columns [14]. In addition, the temperature range *i.e.* volatility of the entire sample has to match the temperature limits of the columns selected for use. Special notice has to be given to the temperature limits of the second dimension column, as its upper temperature limit is usually lower than that of the first dimension column. The maximum allowable temperature of the polar second dimension column determines the upper limit of boiling point of samples that can be analysed by a GC×GC system. This is especially the case when the two columns are housed in the same oven. If the temperature exceeds the higher temperature limit of the second dimension

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column, the column starts to bleed and gives a distorted base line interfering with trace component detection in the sample [11].

In an orthogonal GC×GC system the retention times on the two columns depend on different solute characteristics. In the first dimension solutes are separated according to their vapour pressure differences. The retention times of the polar second dimension column are only determined by chemical class membership. Polarity and molecular shape differences in components of a sample determine retention in the second dimension, making secondary column retention a measure of these molecular properties. That is, the second dimension separates sample components on the basis of their homologous series independent of molecular size or volatility providing a type of functional group analysis. Components of the same homologous series have approximately the same second dimension retention times [13, 14].

The two columns in comprehensive two-dimensional systems may be housed in either of two ways. The two columns can be operated in a one main oven system. Alternatively, an auxiliary oven is used inside the main oven to house the second dimension column. Generally, comprehensive two-dimensional gas chromatographic analyses are performed in a temperature programmed mode. In the one oven system the two columns are operated under the same temperature-programming rate. If a two oven system is utilised it is possible to independently temperature program the two columns. The later case has some advantages [14]:

- The two columns can be operated independently at different temperatures and/or temperature programming rates.
- Columns of high temperature phases can be coupled with columns of limited thermal stability. The overall temperature limit of the system is no longer determined by the phase with the lower temperature limit.
- Optimisation of stationary phase selectivity by temperature adjustment of two sequentially coupled columns, coated with different stationary phases, is easily accomplished.

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3.2.1 Advantages and Shortcomings of Comprehensive Two-Dimensional Gas Chromatography

Comprehensive two-dimensional gas chromatography, compared to linear gas chromatography, has some distinct advantages. The main advantages can be summarised as follows:

1. By virtue of the multiplicative effect of separation space available, comprehensive two-dimensional GC provides very high n_c. This high n_c is directly reflected in the ability of the system to separate complex mixtures effectively. It allows a complex sample to be separated into individual peaks. The peaks are classified into groups creating an easy group type analysis [10, 12, 15].
2. Due to the orthogonal nature of the two dimensions, GC×GC offers two totally independent retention times. These independent retention times increase the certainty of qualitative identification of components [10, 12].
3. It provides increased sensitivity and superior resolution relative to conventional GC. The pulsing of components from the first dimension to the second dimension creates narrower bands which are much taller in magnitude and this translates to a much more sensitive analysis due to the increase in peak height. If a narrow band can be pulsed into a short column leading to the detector, the response can be significantly increased. This allows easy detection and determination of trace components in a complex mixture. A short second dimension column with different stationary phase helps to resolve peaks unresolved in the first column. A fast second dimension column, eluting peaks at a rate faster than the first dimension peak widths, generates a sequence of second-dimension chromatographic separations that increases the overall resolution power of the system [15, 16].
4. Analysis times are comparable with one-dimensional GC, but GC×GC runs contain more information and have superior resolution [8, 12].

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Comprehensive two-dimensional gas chromatography also has a few shortcomings [12]:

1. Limited availability of fast detectors. The only readily usable detectors presently are the mass flow sensitive detectors like the flame ionisation detector. Conventional mass spectrometers cannot be used as GC×GC detectors, as they are too slow to record a spectrum during the elution of very narrow second dimension peaks. The first generation commercial instruments with fast time-of-flight mass spectrometers have only recently become available.
2. GC×GC systems are sometimes inadequate for the separation of high boiling components, often exceeding the temperature limits of the second column. This is especially the case in one-oven systems.
3. The lack of easily accessible data handling software for the automated interpretation and easy processing of the large amount of data produced. The first automated peak integration software package has only recently come onto the market.

3.3 Coupled Gas Chromatographic Techniques for the Analysis of Essential Oils

The analysis and research in herbal plants and their essential oils is becoming increasingly important. This is due to their tremendous applicability not only in traditional herbal preparations but also due to the great demand for essential oils as raw materials in the pharmaceutical and cosmetic industries. The analyses of essential oils aim at one or all of the following: component identification, component comparison between different oils, or for authenticity and quality control purposes. To achieve any of these goals one needs a suitable method or combination of methods, which is able to give the best result in a reasonable time and cost. Two-dimensional methods for essential oil analysis include: capillary gas chromatography-mass spectrometry (GC-MS), supercritical fluid chromatography-gas chromatography (SFC-GC), and comprehensive two-dimensional gas chromatography (GC×GC).

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3.3.1 Gas chromatography-Mass Spectrometry (GC-MS)

GC-MS is a coupled technique combining two independent systems. The first dimension, the gas chromatograph, disperses solutes according to their vapour pressure differences. The second dimension in GC-MS, the mass spectrometer, acts as a detector but with added independent resolving power. It ionises and fragments sample components dispersing them along a secondary mass-to-charge ratio axis [13].

Essential oils have a complex chemistry consisting of terpene hydrocarbons, alcohols, aldehydes, and esters [17]. The components range from highly volatile to semi-volatile and from non-polar to highly polar organic components. The use of gas chromatography as a separation tool is therefore ideal for essential oils. The use of mass spectrometry for the characterisation and detection of the separated solutes follows logically. This offers the reason why most essential oil analyses are done with this coupled technique.

The complete identification of essential oil components is possible with the use of known standards, use of literature retention indices or by comparison with mass spectrometric databases. The use of authentic standards is mostly useful when the analyst has an idea about the composition of the sample at hand. On the other hand, the use of retention times and Kovat's indices requires the use of the same analytical conditions, including same temperature programming rate, column dimensions, stationary phase and flow rates, as the reference data. The use and success of spectrometric databases depends on the quality and amount of data stored. Problems arise when analysed samples contain components not included in the database.

Almost all the studies dealing with essential oils employing GC-MS techniques in the scientific literature focus mainly on the identification of the oil components. Only few publications deal with the factors affecting oil yield and concentration variation of some oil components. Most of these studies were done with similar columns and analytical conditions. Amongst the many studies on essential oils using GC-MS, there are quite a few references that deal with the analysis of the essential oils studied in this project. The GC-MS studies of

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Tagetes minuta indicated that the essential oil contains 22 - 35 identified components [18, 19, 20, 21, 22, and 23,]. The chemical composition of lemongrass consists of more than twenty (20) identified components [24, 25, 26 and 27]. The essential oil of Bourbon Geranium has about thirty (30) identified components [27]. In the case of *Artemisia afra* (African Wormwood) forty-eight (48) components have been identified [28].

3.3.2 Supercritical Fluid Chromatography-Gas Chromatography

The coupling of supercritical fluid chromatography with gas chromatography is one of the alternatives that can be employed to enhance n_c of a chromatographic system and to significantly improve the resolution of individual components in a sample. One of the attractive features of supercritical fluid chromatography (SFC), which makes it ideal for coupling to a fast gas chromatographic system, is its ability to handle low volatility and thermally labile organic components. Supercritical fluid chromatography's compatibility with non-volatile and thermally labile compounds comes from the solvation characteristics of the mobile-phase, supercritical fluid CO_2, at low temperatures. The solvent strength of the supercritical fluid CO_2 is used to pre-separate classes of solutes (hydrocarbons, alcohols, esters and aldehydes) that can be selectively or comprehensively introduced into a high-resolution capillary GC for detailed fingerprinting or quantitative analysis. Therefore, in the first dimension the sample components are subjected to a group-type separation in the polar silica gel SFC column, while in the second dimension they are further separated according to their boiling point by the fast GC column. At the SFC-GC interface the mobile phase is decompressed into CO_2 gas, allowing easy replacement with better GC mobile phases (hydrogen, helium). As compared to the fundamentally similar normal phase LC-GC coupling, no solvent evaporation techniques are required in SFC-GC, as the CO_2 lacks detector response in the flame ionisation detector of the GC.

The use of an in-house built [29] comprehensive two-dimensional SFC and fast temperature programmed gas chromatograph (SFC×GC) for the analysis of the four South African essential oils studied in this project has been reported [30]. The system comprised of an SFC system coupled to a fast GC by a flow modulator that samples every peak from the SFC and re-focuses it into the GC injector using pressure drop focussing. There is a T- junction

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(splitter) after the stop-flow modulator, which splits the SFC flow into the FID of the SFC and directs the rest of the flow into the fast GC injector. The CO_2 from the SFC is replaced by hydrogen as a GC carrier gas. The GC column is resistively heated and rapidly cooled by liquid CO_2 in a one-minute cycle [30].

Comprehensive SFC×GC analysis of essential oils has only been done in our laboratory [30]. Other reports on this coupled technique deal with heart-cutting methods instead. T. Yarita *et al.* [31] reported the use of a heart-cut SFC-GC system for the analysis of some citrus essential oils, *viz.* lemon, grapefruit and orange essential oils. To interface the SFC to the GC, a switching valve was placed between the SFC UV detector and a restrictor to split the CO_2. Then part of the flow was introduced into the GC injector *via* a capillary tube. Helium was used as a GC mobile phase and the components were cryo-focused just above the GC column using liquid CO_2. These authors have demonstrated the capability of the system to efficiently separate the essential oil components into their respective classes, first according to their polarity in the SFC and then their boiling points in the GC. A very similar set-up reported by P. Manninen and H. Kallio [32] was used for the analysis of volatiles in the edible oil of cloudberry seed oil. Here the authors utilised the power of the SFC to separate the volatile components of the oil, which were introduced into the GC after being cold-trapped and re-focused. The low volatility compounds that eluted later were led directly to the FID of the SFC and not introduced into the GC. The authors concluded that this method is very successful in the characterisation of edible oil volatiles [32].

3.3.3 Comprehensive Two-dimensional Gas Chromatography (GC×GC) and the Analysis of Essential Oils

Compared to one-dimensional chromatographic techniques, comprehensive two-dimensional gas chromatography (GC×GC) gives an unmatched separation capability and larger n_c. As in SFC×GC, this technique offers an orthogonal separation of sample components by the use of two serially coupled columns. The solutes are separated according to their volatility in the first dimension and the second polar or chiral column is used for polarity or chirality separation, respectively (the experimental principles and instrumentation of GC×GC will be

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dealt with later on). This option makes GC×GC a much sought after tool for the analysis of complex samples, including essential oils.

Since its inception more than a decade ago, comprehensive GC×GC has been widely used for the analysis of complex petroleum products. Its applicability to other samples, especially essential oils, seems to be gaining momentum in the last three to four years [33, 34, 35, and 36]. The analysts aim at the identification of sample components of different essential oils, separation of enantiomers and more complete fingerprinting of essential oils of commercial and industrial value.

In this study, comprehensive two-dimensional gas chromatography was used to analyse the four South African essential oils described in Chapter 2. GC×GC analysis of these essential oils has not been reported before.

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