UNIVERSITY OF TASMANIA

HIGH-RESOLUTION GAS CHROMATOGRAPHY for ANALYSIS of PLANT EXTRACTS

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Declaration

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of the my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Statement of Co-Authorship

The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

Paper 1: B. Savareear, R.A. Shellie, Multiplexed dual second-dimension column comprehensive two-dimensional gas chromatography (GC × 2GC) using thermal modulation and contra-directional second-dimension columns, *Analytica Chimica Acta* 803 (2013) 160-165.

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Details of the Authors roles:

B. Savareear performed laboratory work. Both authors contributed to experimental design and manuscript preparation.

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We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

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Abstract

This thesis focuses on new high-resolution gas chromatography (GC) techniques to separate complex mixtures, especially plant extracts. First, serially-coupled high-resolution capillary columns are investigated for single-dimensional GC separation of plant extracts. The first research chapter highlights the potential and indicates the limitations of high-resolution one-dimensional analysis of complex plant extracts.

Comprehensive two-dimensional GC (GC × GC) is an extended version of single-dimensional GC that offers exceptionally high peak capacity. This thesis employs a commercially available dual stage GC × GC modulator as a platform for exploring new high-resolution GC techniques. To this end, a multiplexed dual-secondary column comprehensive two-dimensional gas chromatography approach (GC × 2GC) designed for analysis of complex mixtures is introduced. GC × 2GC with a single detector and two second-dimension columns is made possible by the development of contra-directional modulation. Contra-directional modulation leads to two single two-dimensional chromatograms in a single window for each injection. A selection of different classes of compounds is analysed to demonstrate the approach. The chromatogram from this single detector system provides complementary information due to the unique selectivity of the three separation columns and the result is similar to GC × 2GC with multiple detectors previously described in the literature.

Next, a new multiplexed dual first-dimension comprehensive two-dimensional gas chromatography approach (2GC × GC) is introduced. 2GC × GC is achieved by installing the two first-dimension columns contra-directionally through a dual-stage modulator. This study is divided into two parts: the first part discusses the benefits of 2GC × GC analysis compared to normal GC × GC and GC × 2GC analysis with a

similar column configuration. In the second part, 2GC × GC is coupled to mass spectrometry for the characterisation of Australian tea tree (*M. alternifolia*) essential oil.

The concept of dual channel GC is also explored. A multiplexed dual channel GC using a single detector system (2GC- MS) is introduced. This study utilises polar and non-polar columns for the analysis of parsley essential oil. A comprehensive analysis strategy using mass spectrometry and multiple linear retention indices using 2GC-MS is discussed.

Finally, an ultra-dimensional separation technique called "multiplexed dual first and second-dimensions column comprehensive GC × GC (2GC × 2GC) or 2(GC × GC)" with a single detector system is introduced. A combination of non-polar × polar and polar × non-polar separation columns is utilised for separation of parsley and hop essential oils to demonstrate the approach. The dual two-dimensional chromatograms from this single detector system provide complementary information due to the selectivity differences of the four separation columns.

Journal Papers & Conference Presentations from this Thesis

1. B. Savareear, R.A. Shellie, Multiplexed dual second-dimension column comprehensive two-dimensional gas chromatography (GC × 2GC) using thermal modulation and contra-directional second-dimension columns, *Analytica Chimica Acta* 803 (2013) 160-165.

2. B. Savareear, M.R. Jacobs, R.A. Shellie, Multiplexed dual first-dimension comprehensive two-dimensional gas chromatography – mass spectrometry with contra-directional thermal modulation, *Journal of Chromatography A* 1365 (2014) 183-190.

3. B. Savareear, R. A. Shellie**,** E.F. Hilder, *Investigation of Tasmannia Lanceolata Oil using High Resolution Gas Chromatography*, 18th Annual RACI Environmental and Analytical Division R&D Topics conference, University of Tasmania, 5th -8th December 2010.

4. B. Savareear, E. F. Hilder, R. A. Shellie, *Novel comprehensive GC ×2GC approach for complex mixtures separation*, 11th Asia Pacific International Symposium on Microscale Separations and Analysis, University of Tasmania, 27th -30th November 2011.

5. B. Savareear, R.A. Shellie, *Multiplexed dual-secondary column comprehensive two-dimensional gas chromatography*, Separation Science 2012, Kuala Lumpur, Malaysia 25th -27th June 2012, Awarded Silver Poster Prize.

6. B. Savareear, R.A. Shellie and E.F. Hilder, *Multiplexed dual-secondary column comprehensive two-dimensional gas chromatography*, 20th Annual RACI Environmental and Analytical Division R&D Topics conference, Deakin University 11th – 14th December 2012.

7. R.A. Shellie, B. Savareear, *A new twist in GC ×2GC*, 9th GC×GC Symposium, Riva del Garda, Italy 27th May -01st July 2012.

8. B. Savareear, L. Tedone, R.A. Shellie, *GC × 2GC and 2GC × GC using contra-directional thermal modulation*, 10th GC×GC Symposium, Palm Springs, United States 12th -16th May 2013.

9. B. Savareear and R.A. Shellie, *Multiplexed dual-column comprehensive two-dimensional gas chromatography (2GC × GC) for the analysis of Tea tree essential oil*, 13th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology, Bruges, Belgium 29th -31st January 2014.

10. B. Savareear and R.A. Shellie, *Multi-Multi-Dimensional Gas Chromatography*, 11th GC×GC Symposium, Riva del Garda, Italy 18th-23rd May 2014.

List of Abbreviations

$^{1}t_R$	First Dimension Retention Time
2GC	Multiplexed Dual Channel Gas Chromatography
2GC × 2GC	Multiplexed Dual First and Second-Dimension Columns Comprehensive Two-Dimensional Gas Chromatography or Twin Comprehensive Two-Dimensional Gas Chromatography
2GC × GC	Multiplexed Dual First-Dimension Column Comprehensive Two-Dimensional Gas Chromatography
$^{2}t_R$	Second Dimension Retention Time
CO_2	Carbon Dioxide
d_f	Film Thickness
ECD	Electron Capture Detector
FAME	Fatty Acid Methyl Esters
FFNSC	Flavour and Fragrance Natural and Synthetic Compounds
FID	Flame Ionisation Detector
FS	Fused Silica
FTIR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
GC × 2GC	Multiplexed Dual Second-Dimension Column Comprehensive Two-Dimensional Gas Chromatography
GC × GC	Comprehensive Two-Dimensional Gas Chromatography
GC^3	Comprehensive Three-Dimensional Gas Chromatography
H	Plate Height
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
I	Retention Index
i.d.	Internal Diameter
L	Length

LMCS	Longitudinally Modulated Cryogenic System
LN_2	Liquid Nitrogen
LRI	Linear Retention Index
MDGC	Multidimensional Gas Chromatography
M_R	Modulation Ratio
MS	Mass Spectrometry
n	Peak Capacity
N	Efficiency (Number of Plates)
NCD	Nitrogen Chemiluminescence Detector
NIST	National Institute of Science and Technology
NMR	Nuclear Magnetic Resonance
NPD	Nitrogen Phosphorous Detector
PCB	Poly Chlorinated Biphenyl
PLOT	Porous-Layer Open-Tubular
P_M	Modulation Period
qMS	Quadrupole Mass Spectrometry
R_s	Resolution
SCD	Sulphur Chemiluminescence Detector
SCOT	Support-Coated Open-Tubular
t_M	Void Time
TOFMS	Time of Flight Mass Spectrometry
t_R	Retention Time
$\bar{u}$	Average Linear Velocity of Carrier Gas
WCOT	Wall-Coated Open-Tubular
ω	Width at Half Height or at Base

k	Retention Factor
PARAFAC	Parallel Factor Analysis
GRAM	Generalized Rank Annihilation Method
PCA	Principal Component Analysis
PLS-DA	Partial Least-Squares Discriminate Analysis
τ_z	Dimensionless Sampling Period

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Chapter 1

Introduction

1. 1. Background

Gas Chromatography (GC) is a separation technique used for the separation of volatile and semi-volatile substances. In GC the mobile phase carries the vapourised components through a capillary column where differential migration occurs according to components' interaction with the stationary phase. Retention of an individual solute is influenced by the chemical structure of the solute, the characteristics of the stationary phase, the column temperature, and column dimensions.

The combination of speed, sensitivity, and a high resolving power in GC provides an adequate technique for the separation of complex mixtures. Moreover, the coupling to spectrometric methods such as mass spectrometry (MS) for direct identification of unknown compounds is easy to establish. Complex mixtures such as petrochemicals, natural products, environmental and biological samples may contain many hundreds to many thousands of individual components. Highly efficient GC approaches are required to obtain adequate information about such samples. The need for high resolution GC to separate complex mixtures has led gas chromatographers to develop dual channel or multidimensional capillary column GC separation techniques. Although such approaches have provided improved knowledge, chromatographers must still search for more efficient techniques in GC to know more about the chemical composition of these complex mixtures.

Peak capacity

Peak capacity is a very useful measurement of performance of any temperature programmed or multidimensional GC system. Peak capacity (n) is defined as the maximum number of component peaks that can be packed side by side into the available separation space at a given resolution [1]. Unit resolution $R_s = 1.0$ is usually

used for determining peak capacity [2]. **Figure 1.1** shows a pictorial representation of peak capacity for different GC systems.

For a single column analysis (*Figure 1.1a)*

$$n = \frac{\Delta t}{\omega R_s} \tag{1.1}$$

Where Δt is difference in retention time between two adjacent peak t_1 and t_2 ($\Delta t = t_2 - t_1$) and ω is its peak width at half height or at base (ω_b or ω_h).

For the heart-cutting MDGC experiment (*Figure 1.1b*)

$$n = \sum n_i = m.\bar{n} \tag{1.2}$$

Where m = the number of second dimension separations performed, of average capacity $\bar{n}$.

For the comprehensive two-dimensional GC (GC × GC) experiment (*Figure 1.1c)*

$$n_{max} = n_1 \times n_2 \tag{1.3}$$

It is the product of average peak capacity obtained from the first- and second-dimension separations.

Overall GC × GC offers greatly enhanced peak capacity, although conventional heart-cutting MDGC is more powerful in providing resolution enhancement for specific regions of a sample. In a typical GC × GC experiment $n_2 << n_1$, because the second dimension column is much shorter than the first dimension column and it is generally operated using a higher than optimal carrier gas flow rate.

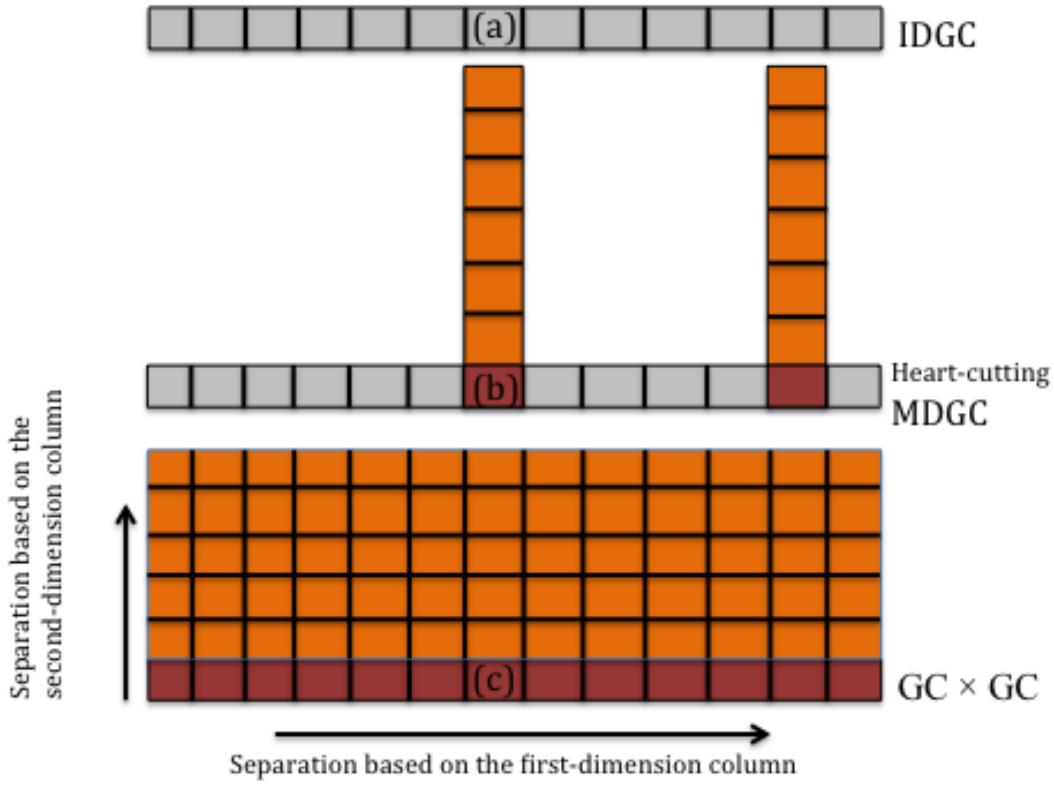

Figure 1.1. Pictorial representation of peak capacity for different GC systems.

Retention time and retention indices

Retention time (t_R) is defined as the time that elapses between injection of a solute in a GC column and elution of its peak maximum [3]. The retention time will depend upon several factors such as geometry of the column, operation parameters, and interactions of analytes between mobile and stationary phases.

Retention times are used for calculating retention indices (*I)* first introduced by Kovats in 1958 [4] using the two *n*-alkanes of a homologous series that are eluted before and after the analyte. Retention time and retention index are very useful indicators of peak identity in a chromatogram. Whilst there have been numerous methods described for measuring relative retention data, the most suitable for temperature programmed GC is the generalisation of the Kovats retention index system by van del Dool and Kratz [5]. Firstly fixed reference points are obtained by attaching the retention index (*I*) to each *n*-paraffin hydrocarbon, where

$$I = 100\ n \tag{1.4.}$$

Secondly the retention index (*I*) for any analyte (X) is determined using *Eq. 1.5*

$$I = 100i\left[\frac{X - M_{(n)}}{M_{(n+1)} - M_{(n)}}\right] + 100n \tag{1.5}$$

Where *i* is the difference in numbers of carbon atoms of the reference materials, and $M_{(n)}$ and $M_{(n+1)}$ are the reference materials bracketing *X*, which have *n*, and *n* + 1

carbon atoms. In linear temperature programmed GC either the retention temperatures or the adjusted retention times are substituted for X, $M_{(n)}$, and $M_{(n+1)}$.

1. 2. High resolution single dimensional GC

Developments in GC column technology, instrumentation, and operational conditions have enabled study of many real world samples in great detail. GC instrumentation typically contains one injection port, one column and one detector port. Initial high-resolution GC separations were performed using capillary columns made with glass material [6,7]. The major breakthrough in high resolution GC occurred since the introduction of fused silica (FS) capillary columns in 1979. Due to their ease of handling [8] they are ideally suited for coupling with MS. These capillary columns with wall-coated open-tubular (WCOT), porous-layer open-tubular (PLOT) and support-coated open-tubular (SCOT) phases offer a much higher efficiency than the classical packed columns in short analysis time [9].

Benefits of high resolution GC

The Golay equation (Eq. 1.6) illustrates that high-resolution GC separation is usually obtained by increasing column length or decreasing column diameter. A combination of both that will generate maximum number of theoretical plates, and thus highest peak capacity.

$$H = \frac{2D_G}{\bar{u}} + \frac{\left(1+6k+11k^2\right)r^2}{24\left(1+k\right)^2 D_G}\bar{u} + \frac{2kd_f^2}{3\left(1+k\right)^2 D_L}\bar{u} \qquad (1.6)$$

Where D_G is diffusion of solute molecule in the mobile phase; D_L is diffusion of solute molecule in the liquid phase; $\bar{u}$ is linear velocity of the mobile phase; r is

internal diameter of the column; d_f is film thickness of coating stationary phases and k is retention factor.

Theoretically, a standard 50 m × 0.25 mm i.d. capillary column will give 200,000 theoretical plates ($N = L/r$) and will provide a peak capacity of several hundreds ($n \alpha \sqrt{N}$). Berger [10] produced more than 1.3 million effective plates using a 450 m × 0.20 mm i.d. × 0.33 μm d_f for the analysis of a gasoline sample. This study coupled nine 50 m individual columns in series, making a 450 m long capillary column. This approach was able to resolve 720-970 peaks in a mixed gasoline sample in a 650 min analysis time. Johansen [11] analysed the gasoline sample by employing a 105 m long column consisting of 100 m of a 250 μm d_f methylsilicone column with a 5 m long 5% phenyl pre-column able to separate approximately 350-400 components in less than a 110 min analysis time.

In 1984, Mullin and co-workers [12] used a high-resolution 50 m capillary column (0.20 mm i.d.) coated with 5% phenyl 1% vinyl methyl polysiloxane column for the analysis of 209 Polychlorinated Biphenyls (PCBs) congeners. A 140 min temperature program was able to separate 187 PCB congeners out of 209 PCBs, with only 11 pairs of compounds not fully resolved. Recently Sandra and co-workers [13] employed an extremely efficient GC capillary column for the separation of 209 PCBs. A narrow-bore 80 m × 0.10 mm i.d. × 0.10 μm d_f film of 5% diphenyl 95% dimethyl polysiloxane stationary phase produced peak capacity of 1198 and a peak capacity of 724 . This GC system coupled with mass spectrometry (MS) was able to resolve 195 PCB congeners in a 96 min separation time, using spectral deconvolution and chemometric procedures.

To date the longest capillary column used for the plant extract analysis was 100 m long and was able to generate 400,000 theoretical plates [14]. While the relatively low complexity of sage essential oil may not necessitate the need for an ultra-high resolution column, the authors performed the analysis using this column and were able to assign a total of 45 individual compounds present in the sage essential oil [14].

Limitations of single-dimensional GC

The high-resolution separation of complex mixtures using a single capillary column often lacks the necessary resolution power to isolate all matrix components in an acceptable analysis time. GC theory suggests that, to generate the necessary peak capacity by manipulation of either column length or column diameter or a combination of both of these to improve high-resolution in GC separation [13]. The longest capillary column ever produced was 2,100 m × 0.32 mm i.d. × 0.1 μm d_f of dimethyl polysiloxane stationary phase of which a 1,300 m length of this column generated 2×10^6 theoretical plates [15]. Although the plate number is outstanding the limitation of such a column is need for high inlet pressure and longer analysis time [15]. Giddings predicted that peak resolution can only be generated using up to 37% of the peak capacity, and the number of single component peaks in a chromatogram cannot exceed 18% of the peak capacity in order for (95%) chance of all being resolved [16]. Later Davis and Samuel discussed this overlapping of peaks in single dimensional GC using statistical overlap theory for a range of real-life complex mixtures [17]. Complete analysis of complex real-life samples is beyond the theoretical and practical capabilities of one-dimensional analysis. While another option to improve resolution in complex mixtures separation is use of selective stationary phases [18], the selectivity benefits are less apparent for genuine multicomponent samples.

To enhance the selectivity and resolution in complex mixtures especially plant extract analysis, the separation is often performed using two complementary stationary phases [14-17]. Different stationary phases exhibit different mechanisms, a polar stationary phase column interacts mainly through the activity coefficient (polarity) and non-polar stationary phase column mainly through the dispersive forces (boiling point). It is also very valuable when a retention index, *I* is obtained from two distinct stationary phases to enhance confidence in assignments for the identification of unknowns in plant extracts [19-21]. Bicchi and co-workers [22] pointed out that if a suitable library reference *I* record is available, the percentage of correct identifications obtainable through *I* is approximately 65%, 80% and above 90% with one stationary phase, two different-polarity columns and three columns respectively. A high-resolution separation of peppermint oil was reported using three distinct capillary columns: 100% methylsilicone (50 m × 0.25 mm i.d.); polyethylene glycol (50 m × 0.25 mm i.d.) and 100% dimethyl polysiloxane (50 m × 0.25 mm i.d.). The authors reported that the *I* for these three stationary phases for temperature programmed analysis showed the differences of *I* behaviour in each stationary phases [9].

These multiple stationary phase analyses not only facilitate the calculation of *I* information but also provide an extra separation power of target analytes. Shellie and Marriott [23] studied Australian tea tree oil (*Melaleuca alternifolia*) and other *Melaleuca sp.* oils with an MS library search combining interactive use of multiple *I* on polyethylene glycol and 5% diphenyl 95% dimethyl polysiloxane columns (both: 30 m × 0.25 mm i.d. × 0.25 μm d_f) respectively. This study reliably characterised 80% of the detected sample components and showed that some important oil constituents were co-eluted on a non-polar column, which were completely resolved by a polar column. For instance the co-elution of *cis*-sabinene hydrate and linalool; viridiflorene

and bicyclogermacrene; and 1,8-cineole, limonene and *p*-cymene on a non-polar column were completely resolved using polar column, facilitating correct identification and quantification. A similar kind of analysis was presented very recently by Tedone and colleagues [14] for the characterisation of sage essential oil using 100 m long polar and non-polar capillary columns and showed the co-elution of some important compounds with a 30 m column able to be completely resolved by the highly efficient capillary columns.

Confident identifications of unknown compounds from a plant extracts can be confirmed by two or more *I* from different stationary phases with a combination of mass spectral data. This requires multiple analyses, and thus boosts the cost and analysis time. Consideration of the mutual relationship between speed, resolution and sample capacity, a fast GC method used for reducing analysis time in plant extract analysis is not a recommended approach for high resolution separation with trace level unknowns. To overcome all these aforementioned drawbacks such as peak capacity, selectivity and analysis speed associated with single dimensional GC led researchers to develop alternatives. These approaches will be described in the following sections.

Dual channel GC analysis

The term "dual channel gas chromatography" refers to a special type of gas chromatography analysis. The instrument set up of dual channel GC is shown in **Figure 1.2**. The sample is introduced through an injection port and the flow split into two different capillary columns that terminate at the end of two similar or different detectors. This kind of instrumentation provides two chromatograms from a single run and offers more than twice the qualitative and quantitative information that is

available in a single channel system. The ultimate aim of any dual channel analysis is to save analysis time by obtaining *I* from two different stationary phases in a single run.

The combination of polar and non-polar column in dual channel analysis has been rapidly accepted for the analysis of essential oils. A dual channel analysis of lime oil using a methylsilicone and polyethylene glycol coated capillary column was reported and its unique complementary nature of the separation was shown [9]. The authors also noticed the polar column was not good for the separation of hydrocarbons such as limonene and pinene which are poorly retained. On the other hand, such a column resolved well the oxygenated compounds such as linalool and geraniol. While there were no clues given as to the functionality of the non-polar column it may be correlated with boiling points [9]. These differences of stationary phase selectivity are also experienced in conventional single-dimensional GC analysis using polar and non-polar columns run individually [14,23-25].

Chatzopoulou and co-workers [26] analysed *Achilleamillefolium L.* essential oil using 100 % dimethyl polysiloxane and polyethylene glycol (both: 60 m × 0.25 mm i.d. × 0.25 μm d_f) capillary columns with dual channel gas chromatography. The two columns were connected in the injector port by means of a twin hole ferrule that spilt the sample equally between the two capillary columns. The identification of compounds was carried out by comparison of their mass spectra and *I* on the two columns of different stationary phases, which provided identification of 56

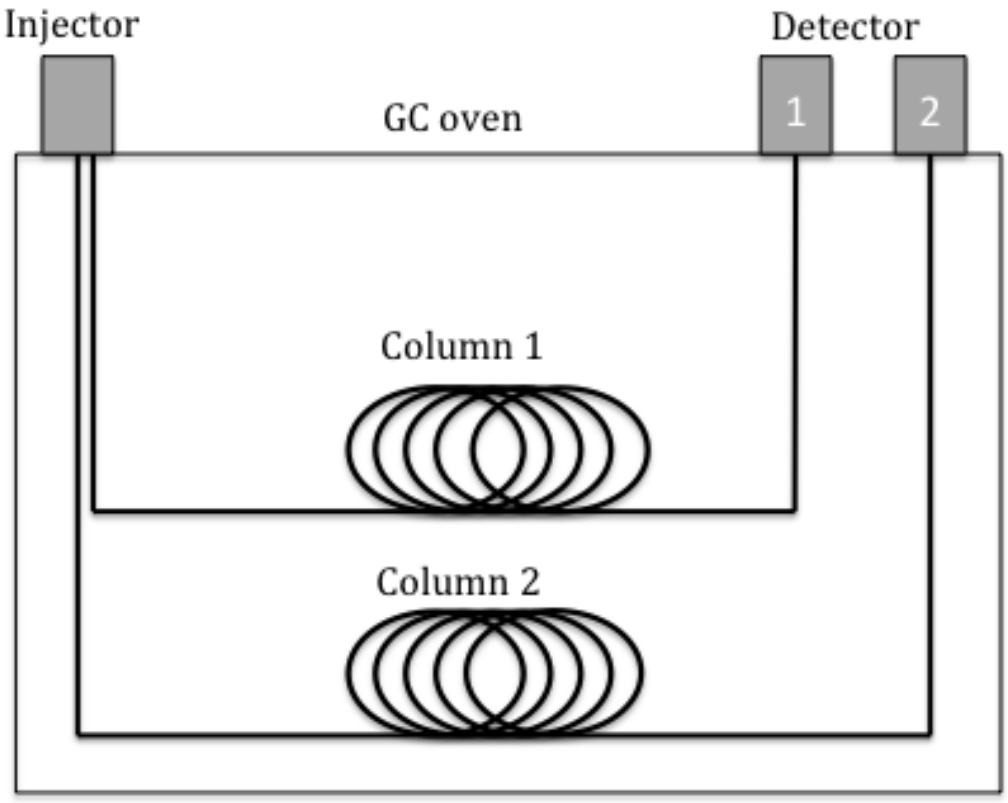

Figure 1.2. Schematic diagram of dual-channel GC instrumentation.

compounds present in the sample. A similar kind of study was applied on different essential oils: four essential oils obtained from Greek oregano clones [27], essential oils of *Lavandula angustifolia* and the Lavandin hybrids "*super*" and "*special*" grown in Greece [28] and essential oils from hop of various *Humulus lupulus* L. cultivars [29].

The majority of these studies utilised two flame ionisation detectors to obtain two *I* from different stationary phases and for the identification run on the same sample with conventional GC-MS analysis. Matching of thus obtained mass spectral data combined with twin *I* from different stationary phases was used for reliable characterisation of plant extracts. The drawbacks associated with these approaches are coupling of two MS. Such an instrumental configuration is problematic and too expensive for most users. Furthermore, data processing is time-consuming from two different detectors. Uneven split may be observed when splitting the effluent into two columns; as a result quantitative analysis will be difficult to perform. However the disadvantages associated with high-resolution GC and dual channel have GC led researchers to find alternative approaches.

1. 3. High resolution multidimensional GC (MDGC)

Using a single column to analyse complex mixtures may produce many overlapping peaks. Thus analyses are frequently performed using more than one dimension to enhance peak capacity. The term multidimensional describes coupling of two or more columns or selective detection techniques. GC-MS or other selective spectrometry detectors are considered multidimensional approaches since they use different analysis approaches in unrelated (orthogonal) dimensions to improve the quality of data from an analysis [30]. MDGC separations often fall into two categories: heart-

cutting MDGC or GC × GC. The difference between the two approaches is, in heart-cutting MDGC only a small (targeted) portion of sample from the first dimension column is transferred to the second-dimension column for further separation. Whereas, in GC × GC the entire sample eluted from first-dimension column is transferred into the second dimension column for further separation.

Multidimensional analysis

GC-MS

The combination of two different analytical techniques is often referred to as multidimensional analysis. In 1959 Gohlke [31] first coupled MS with GC and since then the approach has been widely used for many fields. GC is used to separate the analytes present in a mixture and MS is used to analyse their mass, providing more qualitative information about the sample. GC-MS (when operated MS in electron ionisation mode) is considered as orthogonal two-dimensional analysis [30]. The essential oil industry relies on compound identification using *I* information obtained from GC with mass spectral data obtain from MS. The spectral matching is often aided by the availability of commercial mass spectral libraries, such as Adams, Flavour and Fragrance Natural and Synthetic Compounds (FFNSC), and National Institute of Science and Technology (NIST) [32-34].

There are vast numbers of papers published about the analysis of plant extracts using GC-MS. The characterisation of essential oils using MS alone can result in erroneous identification due to the very similar fragmentation patterns produced by these analytes [35,36]. More reliable identification can be obtained using *I* obtained from GC with correlation of mass spectral data. High-resolution GC combined with MS was used for determining the chemical composition of the plant extracts of marjoram

oil cultivated in Finland [37]. This study used a chemically bonded 100% methylsilicone (50 m × 0.3 mm i.d.) capillary column able to separate 56 compounds; among them 18 compounds were reported for the first time. The unknown compounds were identified through *I* from different-stationary phases such as 100% methylsilicone, 5% phenyl 1% vinyl methylsilicone phase and polyethylene glycol capillary columns with mass spectral data where available.

While GC-MS is classified as a multidimensional analysis approach for qualitative and quantitative determination of plant extracts, the drawbacks of such technique is that the confirmation of compounds often required multiple analyses that boost the total analysis time and cost. Furthermore a single column provides extensive peak overlapping and the structural similarity of many terpene compounds particularly in plant extract analysis is often a hindrance to reliable MS structural elucidation. It is reported that more than 400 sesquiterpenes with many have similar or almost similar patterns of mass spectra [35] and this often leads to false assignment unless adequate separation is performed before mass spectral analysis.

Heart-cutting MDGC

Heart-cutting MDGC was first described more than 50 years ago [38]. This kind of technique usually consists of two conventional capillary columns (e.g., 30 m × 0.25 mm i.d. × 0.25 μm d_f) and these columns are connected in series and characterised by a differing selectivity (e.g., non-polar-polar, polar-chiral etc.). The columns can be located either in a single or two separate ovens [39-41]. The transferring of effluent from the first dimension column into the second dimension column through an interface is generally classified into three approaches (1) in-line valve, (2) Deans switching and (3) valveless system [42].

An on-line heart-cutting MDGC using two separate ovens is developed for the analysis of peppermint and lavender essential oils for determining their enantiomeric compounds present in the samples [43]. An open tubular capillary column of 20 m × 0.53 mm i.d. × 1 μm d_f coated with methyl polysiloxane as primary column and a 12 m × 0.22 mm i.d. 5% phenyl containing nickel (II) bis[3-heptafluorobutanoyl)-(1R)-camphorate] as secondary column was able to resolve menthone, isomenthone and menthol from peppermint oil and stereoisomers of linalyl acetate from lavender oil respectively [43]. Mondello and co-workers [40] developed fully automated heart-cutting MDGC for the analysis of cold pressed lime oils with analysis time more than 60 min. The authors reported the distribution of enantiomers in the targeted region of the chromatogram now fully resolved using 5% phenyl 95% dimethyl polysiloxane (30 m × 0.32 mm i.d. × 0.40-0.45 μm d_f) and diethyl-tert-butyl-β-cyclodextrin (25 m × 0.25 mm i.d. × 0.25μm d_f) capillary columns.

Sciarrone and co-workers [44] reliably characterised different sandalwood oil collected from different geographical origins using a heart-cutting MDGC system with simultaneous MS and FID detection recently. A combination of non-polar and polar capillary column improved the resolution of the compounds of interest through heart-cutting at the complex region of oxygenated sesquiterpenes and compared it with GC-MS results. The coelution of (*E*,*E*)-farnesol with (Z)-*β*-santalol on non-polar column and with (Z)-*α*-santalol on polar column is now nicely separated with the heart-cutting MDGC [44].

It is clear that heart-cutting MDGC has been best suited for applying maximum resolving power to a small number of analytes since only a small region is subjected to the second dimension column separation. This is often performed in the complex or

unresolved region of the chromatogram. A two times heart-cutting MDGC analysis of tobacco smoke resolved approximately 1500 compounds and 300 compounds were identified using mass spectra and retention indices [45]. The secondary chromatogram still contains many unresolved components and this is a situation requiring another separation which is capable of resolving all individual compounds present in a complex mixture. Marriott and co-workers [46-48] developed a targeted MDGC system using cryogenic trapping and a microswitching valve between two GC columns. By using this method, MDGC with multiple heart-cuts can be completed within the same time as the primary column separation. Moreover, this system provides a high-resolution separation in the second-dimension with increasing sensitivity. Full characterisation of a complex mixture in heart-cutting MDGC requires the transfer of a large number of individual cuts. The process becomes laborious and time consuming.

Comprehensive two-dimensional GC (GC × GC)

GC × GC is a version of heart-cutting MDGC in which the entire effluent leaving from the first dimension column is transferred into the second dimension column separation via an interface while preserving the resolution of first dimension. The GC × GC technique was introduced in 1991 by Liu and Phillips [49] and ever since each year research brings improvements and insights that allow a wider range of complex mixtures to be analysed with more informative, high-resolution separations. The instrumental set up of comprehensive GC × GC shown in **Figure 1.3**. The interface known as a modulator transfers the effluent leaving from the first dimension column to the head of the second dimension column as a repetitive series of pulses. In other words it traps the analyte eluted from the first dimension column and then refocuses

and re-injects into a fast second dimension separation usually a few seconds in duration. This process continues until the end of GC run. The first dimension retention time $^{1}t_{R}$ is defined as the time elapsed between injection of the analyte and its arrival to the modulator, while the second dimension retention time $^{2}t_{R}$ is the time taken for the analyte to elute between modulator and detector. $^{2}t_{R}$ will depend upon the modulation period [(P_{M}) is the time used to modulate the primary peak as pulses], modulation mechanism, second capillary column length and other GC operational parameters. Both $^{1}t_{R}$ and $^{2}t_{R}$ information are required for positive identification of compounds in GC × GC analysis.

GC × GC has many advantages over other high-resolution GC techniques described in this chapter. First, the two columns with different stationary phase provided an extra resolution in the overall separation. This extra dimensional information provided more qualitative information about the samples to be analysed. When coupling with a MS in such instrumental configuration provides two *I* information with a mass spectral data makes the system is a most powerful way to perform qualitative analysis. This three-dimensional GC × GC-MS technique offers very rich information about sample components in less time than other high-resolution single-dimensional GC or heart-cutting MDGC approaches, which use long capillary columns to obtain adequate peak capacity. Overall GC × GC-MS is a most powerful tool for complex mixture analysis with more qualitative information than GC-MS or heart-cutting MDGC-MS techniques.

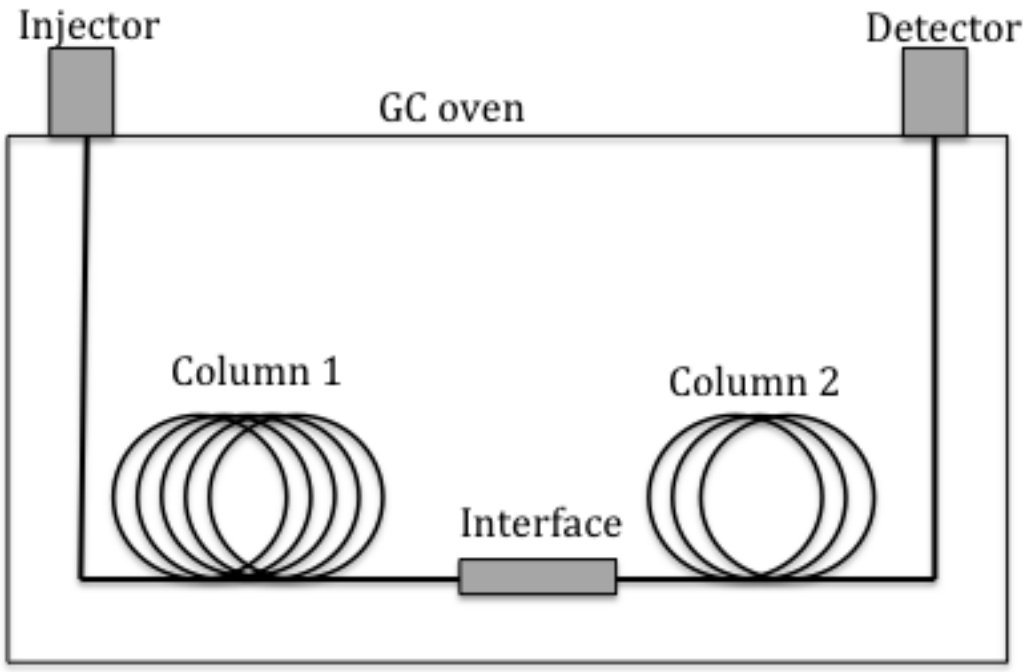

Figure 1.3. Schematic diagram of GC × GC instrumentation. The interface controls the transfer of first-dimension column effluent to the second-dimension column.

Some fundamental concepts, and instrument requirements of GC × GC

Stationary phase and orthogonality

The term orthogonality means two different and independent separation mechanisms must be used in the two GC columns that are connected in series together [50]. In a typical GC × GC system, the first dimension column has a non-polar stationary phase, (usually a dimethyl polysiloxane phase) with a second dimension column whose stationary phase is more polar (usually polyethylene glycol, phenyl methyl polysiloxane, or cyclodextrin derivatives) [51,52]. In such combination the solutes mainly interact as a function of boiling point (dispersion force) in the first dimension, while in the second dimension as a function of activity coefficient (polarity) of the analytes. Although both dimensions are GC, the separation mechanisms are different, which spreads the peaks across the two-dimensional separation space. The orthogonality principle is clearly depicted in **Figure 1.1c**. Components, which are strongly retained in the first-dimension column, might be expected to be strongly retained in the second-dimension column creating a two-dimensional chromatogram with highly correlated retention times [53]. On the other hand, it is very active field of research to measure the orthogonality in GC × GC separation graphically by measuring peak distribution over the two-dimensional separation space [54]. It is reported that better distribution of peak along two-dimensional separation space can be achieved with chromatographic conditions (including stationary phase selection) and characteristics of sample to be analysed [54].

To date most of the GC × GC analyses used a combination of non-polar versus polar column to the respective first and second dimension for complex mixture analysis. Depending upon the applications other possible combinations of columns are also

used. Typically the first dimension column is similar to those used for conventional GC, which is 15 to 60 m long with an inner diameter of 0.25-0.53 mm and film thickness on the order of 0.25-1 μm. These columns can produce conventional peak widths of 5 to 30 s [55].

In order to prevent the so called wraparound effect [53] and maintain high resolution in the second dimension separation space the second column is usually shorter, 0.4 to 4 m long and has a smaller inner diameter of 0.05-0.20 mm and film thickness of the order of 0.1-0.25 μm. Wraparound is often encountered in a GC × GC separation when the second retention time of the compounds exceeds P_M as a result that this compound occurs in the next or subsequent sequences of modulation. The major drawbacks of wraparound are the slight increases of second-dimension peak widths that could ultimately reduce the peak capacity and the possibility of co-elution (or peak overlap) when single dimensional detectors are used. The minimisation of wraparound also can be attained using a secondary heating oven for the second-dimension column. Although narrow bore columns provide high efficiency in separation but one must consider the pressure drop in the second-dimension column. In this respect proper tuning required depends upon the application.

Instrument requirements for GC × GC

Modulation techniques of GC × GC

The modulator is the heart of a GC × GC system and which is an interface situated between two capillary columns in the GC oven (**Figure 1.3**). The function of the modulator is to focus, re-concentrate, and re-inject portions of the first-dimension column effluent onto the head of a second-dimension column as a repetitive series of pulses continuously throughout the analysis. The time required to complete this

process is called P_M and it is generally in the range 1-12 s. The modulator slices the larger peak widths from the first-dimension column into several individual cuts and not only enhances the overall separation efficiency but the refocusing step may also enhance the sensitivity. This sensitivity enhancement is clearly illustrated by comparing non-modulated peak with modulated peak results obtained from GC-FID and GC-MS with GC × GC-FID and GC × GC-time of flight MS (TOFMS) respectively [56].

There are several different types of modulator. Modulators are generally categorised as thermal or pneumatic modulators and some of them are commercially available. The thermal modulators use a positive and/or a negative temperature difference with respect to the GC oven temperature to achieve GC × GC process, while pneumatic modulators use a switching device to manipulate the primary column flow for this purpose. All modulators developed have distinct advantages and disadvantages.

Initially a dual-stage thermal-desorption modulator [49] was reported. Further investigations to improve the modulation step led to development of a rotating thermal sweeper modulator [57] for GC × GC.

Both thermal-desorption and thermal sweeper modulators had many disadvantages. These are not suited for accumulating the more volatile compounds and limit the analysis temperature 100 ^{o}C below the operating temperature of the stationary phase. Moreover, optimisation of operating conditions such as sweep velocity, pause time, thickness of stationary phase, and temperature difference between oven and heater are tedious task and time consuming process [55]. After the introduction of other modulators the number of publications using these modulators declined and it is no longer commercially available [55]. Later several other heating modulators were

designed [58] however these modulators had same drawbacks and there are not many subsequent papers published with these modulators.

On the other hand pneumatic or valve-based modulators using switching devices have been an active field of research for GC × GC separation since 1998 [59,60]. Valve-based modulators are low cost in construction and operation, and can be used for very fast second-dimension separation of 1 s due to production of narrow injection bands in time [55]. In general the disadvantages of valve-based modulators outweigh the advantages. Firstly most of these valves limit the upper operation temperature of the stationary phase to the analysis of semi-volatile compounds. Secondly, only certain percentage of primary column effluent transfers into the second dimension leading to loss of sensitivity. Third, elaborative construction of certain valve-based modulators is time consuming for optimisation of operating condition. Finally, most of them use high secondary flow that prevents the use with MS because of vacuum pumping capacity limitations.

Several researchers modified these valve-based modulators to improve the performance of GC × GC process. Seeley and co-workers [61] modified the six-port diaphragm valve modulator to improve the sensitivity with a stainless-steel sampling loop able to transfer 80% of the primary column effluent into the second dimension. Synovec and co-workers between 2003 and 2004 carried out a series of modulation studies using a high-speed, six-port diaphragm valve, equipped with a low capacity accumulation loop [62-64]. Apart from the limited loop volume and the transfer of only 10% of primary column effluent the benefits are that it can be coupled with TOFMS since low secondary flow, produce very narrow peak widths in the range 100-330 ms, and extended the analysis temperature range up to 270 ^{o}C. Later the same research group developed a 100% primary column effluent transfer modulator

using the same valve, mounted face downwards on top of the GC oven (half-in-half-out) configuration [65]. Seeley's group proposed several improvements in valve-based modulators and one of their design was commercialised by Agilent Technologies [66].

The major breakthrough occurred in the GC × GC separation was the development of cryogenic modulator system. The first cryogenic modulator called longitudinally modulated cryogenic system (LMCS) consists of a moving cryogenic trap and uses liquid CO_2 as a coolant and GC-oven temperature for re-mobilisation of trapped analytes [60]. LMCS provided robust, efficient entrapment, was not restricted by GC-oven temperature limitations, had a less elaborate construction than other existing modulators at the time, and provided a high signal-to-noise ratio [60].

In 2001 Beens and co-workers [67] developed a dual-stage modulator that also focuses analytes with liquid CO_2 as a coolant and GC-oven temperature for re-mobilization of focussed analytes. The authors affirmed that a second-dimension injection bandwidth of 10 ms calculated for C_{14} alkane. Moreover this system does not have a moving part like LMCS, which was listed as an advantage. CO_2 modulators are limited for analysis of more volatile components. Carbon number less than C_7 are difficult to modulate and peak widths are generally greater compared to liquid N_2 plus heating modulators (e.g., loop-type and quad-jet modulators described below) [58].

Based on the study of Ledford and Billesbach, Leco Corporation released a quad-jet dual-stage liquid N_2 modulator and a secondary oven under the licence from Zoex Corporation [55,58]. The dual-stage process of this modulator designed with two jets able to spray cold (liquid N_2) and hot (warm N_2) air pulsed in an alternating mode. The simultaneous activation of quad-jet modulator process produced very narrow,

second-dimension pulses. The mechanism of this modulator is described in Chapter 3, 4, 5 and 6 of this thesis. The ability of liquid N_2 goes down to -190 ^oC that enables to analyse highly volatile compounds like propane and butane. More recently Leco Corporation reduced the consumption of liquid N_2 by introducing a cooled-chilled air for cooling, which enable to trap carbon number between C_7-C_{55} [68].

In 2002, Ledford and co-workers [69] developed a loop-type modulator to reduce the consumption of liquid N_2 and that essentially works same as quad-jet dual-stage modulator system. The two stages are created by looping a segment of capillary column (approximately 1-1.5 m) through the pathway of a cold jet of N_2 gas [69]. Zoex Corporation commercialised this loop-type modulator and more recently and modified the cooling process with refrigeration unit to avoid the consumption of liquid N_2 [70]. It is reported that this refrigeration unit is able to go down to -90 ^oC, which is sufficient for the entrapment of C_7 alkane. The drawbacks associated with these two modulators are they require proper optimisation of delay loop length and the latter is restricted for analysis of highly volatile compounds [58].

Overall the role of any modulator used for GC × GC analysis is periodically transferring the effluent leaving from the first dimension column into the second dimension column separation. To obtain a high resolution in the second dimension separation the first dimension peak should be modulated at least three (modulation in phase) or four times (modulation out of phase) [71]. This will enable correct peak construction in the two-dimensional chromatogram for accurate identification and /or quantification. Seeley [72] explained the effect of sampling duration using low duty cycle modulator such as valve-based modulators with dimensionless sampling period [(τ_z) is the ratio of the modulation period to the standard deviation of the primary peak] for a Gaussian peak. By using this method, GC × GC separations can be produced without a substantial loss in quantitative precision and with only

a moderate loss in first dimension column resolution if the sampling period is less than 1.5 times the primary peak standard deviation. If the sampling period increases above 1.5 times the primary peak standard deviation, this increases the peak broadening and the quantitative precision of total peak areas and first dimension retention time determination are rapidly reduced. Khummueng and co-workers [73] explained the effect of sampling duration using thermal modulator with modulation ratio [(M_R) is the ratio of 4 times the first column peak standard deviation (4σ) divided by the modulation period. The authors outlined that decreasing the value of M_R will decrease the number of modulated peaks. When the M_R value reaches unity or less will give either one (modulation in phase) or two (modulation out of phases) modulated peaks and no more. In addition, the authors also emphasised that the number of observed modulated peaks depends on the peak size and extent of tailing, the phase of modulation and how small a modulated peak is to be measured.

Detector

After the modulation process, bands leaving from the second-dimension column are very narrow (peak width ~60-600 ms). The detector must have a very fast acquisition rate in order to ensure the complete reconstruction of the second-dimension chromatograms. FID is the detector of choice for many GC × GC analysis since it provides very fast acquisition rates typically 50 to 300 Hz. Element selective detectors such as electron capture (ECD), nitrogen- and sulfur-chemiluminescence (NCD and SCD), nitrogen-phosphorous (NPD), and flame photometric (FPD) detectors have acquisition rate typically 50 to 200 Hz have also used for selective applications in GC × GC analysis [55,74-79].

Mass spectrometry detection must acquire data at the rate of at least one mass spectrum every 0.02 s for very narrow pulse generated after GC × GC [55]. This acquisition speed limits use of quadrupole MS (qMS) and high resolution MS

(HRMS) detectors, nevertheless some studies used them by reducing mass-scan ranges to obtain adequate acquisition speed for GC × GC analysis. TOFMS is usually the detector of choice for GC × GC system due to its capability to produce full mass-range spectra at rates greater than 100 Hz. TOFMS has the advantage of producing non skewed spectra because virtually all ions in each MS acquisition period are collected at the same time point of the chromatogram. This ensures that ion ratios remain the same across the GC peaks. This spectral continuity allows mass spectral deconvolution of overlapping GC peaks when the fragmentation patterns of the co-eluted compounds are different [55]. A commercial GC × GC-TOFMS instrument available from Leco Corporation and has proven to be robust enough to be considered as a routine instrument in complex mixture analysis. It has spectral acquisition rate up to 500 spectra/s and mass range from 1 to 1000 u.

Data Acquisition, Visualisation and Data Processing

The needs for data visualisation and data processing methods are different in GC × GC than for single-dimensional GC and heart-cutting MDGC analyses. A schematic representation of data generation and visualisation of GC × GC data shown in **Figure 1.4**. The peaks leaving from the first dimension column undergo a modulation process in which each peak is cut into a series of pulses. Murphy and co-workers [71] recommend that to obtain high two-dimensional resolution, each peak in the first dimension should be sampled at least three or four times. Pulsed chromatograms are often transformed into a 2D representation in which the second-dimension chromatograms are stacked, side-by-side, with each axis representing a particular column retention time. After further treatment, the 2D representation is usually visualised by means of a contour plot or 3D surface plot. This process is clearly

outlined in the literature [55,80-82]. Initially, this was carried out with classical software such as Visual Basic, MatLab and LabView, to create customised programs for data processing. There are now many software packages commercially available that perform the transformation of the pulsed chromatogram into 2D or 3D representations such as ChromaTOF, ChromSquare, GC-image, and Hyperchrom.

The separation of complex mixtures generates hundreds to thousands of individual peaks. The data interpretation for these chromatograms is a challenging task. The Pegasus 4D GC × GC-TOFMS instrument with ChromaTOF software from Leco Corporation has proven perfectly suitable for routine analysis. This software package provides fully automated data processing and instrument control. Stadler and co-workers [83] utilised this system for the application of the decomposition of volatile organic compounds in pig carcasses and utilised the inbuilt statistical comparison tool to distinguish compositional differences between decomposition and control samples, in order to characterise the volatile breakdown products of soft- tissue decomposition. There is ongoing research dealing with the incorporation of chemometrics into GC × GC data handling software, mainly based on mutltivariate analysis including parallel factor analysis (PARAFAC), generalized ranked annihilation method (GRAM), principal component analysis (PCA), partial least-squares discriminate analysis (PLS-DA) and so on. Selecting these chemometric techniques depends on the analysis situation and the information is required to answer any specific analytical questions.

Benefits of GC × GC for plant extract analysis

Although GC × GC has been in existence from 1991 it wasn't used for plant extracts analysis until 2000 when the analyses of spearmint and peppermint essential oils were reported [84]. The authors showed the potential of GC × GC for plant extract

characterisation using a thermal modulator with a non-polar 100% dimethyl polysiloxane column in the first dimension and a low mid-polar 14% cyanopropylphenyl methyl polysiloxane column in the second dimension. The authors also made a critical comparison of their results with GC-MS, showed that the peppermint and spearmint oils had 89 and 68 peaks respectively by using GC × GC, whereas GC-MS only showed 30 and 28 peaks respectively. In addition, they also made a comparison of same compounds identified between these two mint essential oils, using GC × GC showed 52 similar compounds compared to GC-MS that could showed only 18 compounds.

Marriott and co-workers reported the analysis of vetiver [85], and tea tree and lavender [86] essential oils using an LMCS modulator. The analysis of vetiver oil used two sets of columns: one was 5% phenyl polysilphenylene-siloxane × 50% phenyl polysilphenylene-siloxane and the other was 5% phenyl polysilphenylene-siloxane × polyethylene glycol. The authors stated that the 50% phenyl column does not resolve peaks, which may have different polarities of the type, expected in vetiver oil, whereas polyethylene glycol provided the necessary polarity and selectivity to resolve many overlapping peaks. In addition GC × GC revealed more than 200 compounds compared to the same column set run without modulation [85]. In the analysis of tea tree and lavender oils, a non-polar × mid-polar column set under normal GC temperature program rate such as 60-240 ^{o}C at 3 ^{o}C/min was used [86]. GC × GC results were compared with conventional GC using the same experimental conditions and showed the benefits of GC × GC such as improved degree of resolution and sensitivity. Both studies described above confirmed the identification of separated compounds using GC-MS data and *I*.

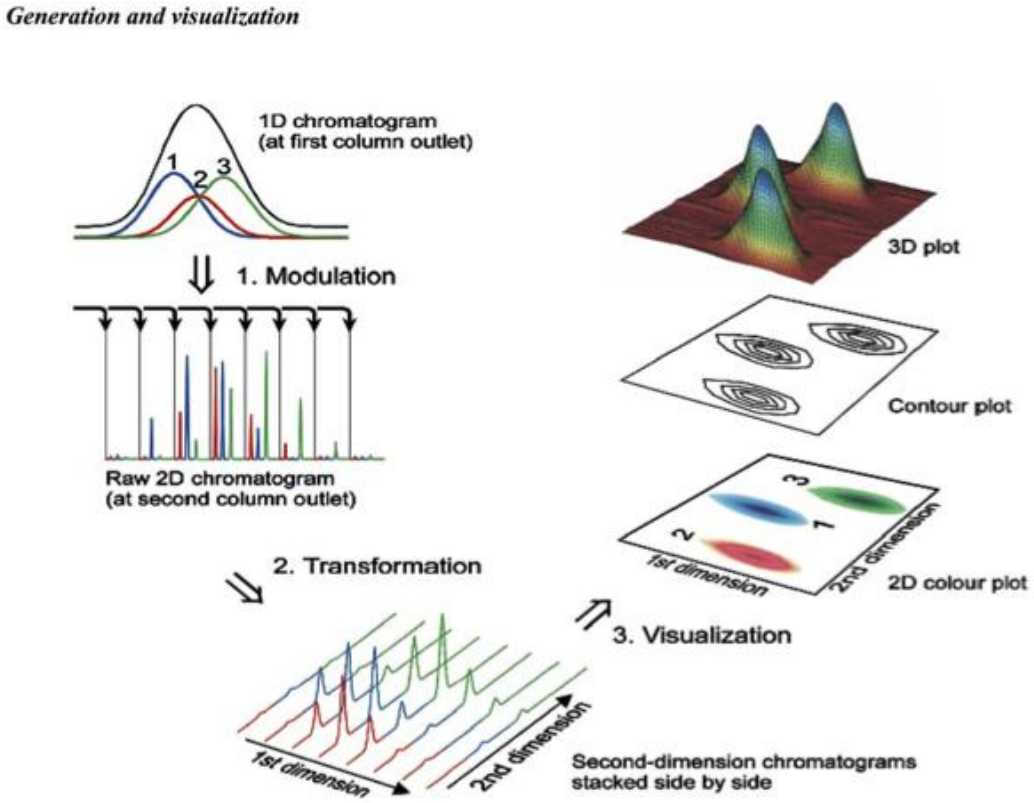

Figure 1.4. Schematic diagram of data generation and visualisation in GC × GC for three overlapping peak emerging from the first dimension column

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In 2001 Shellie and co-workers [87] coupled TOFMS with GC × GC for the analysis of French lavender essential oil. The system consisted of an LMCS cryogenic modulator with non-polar (5% phenyl dimethyl polysiloxane) and polar (polyethylene glycol) columns. The authors stated that triple-dimensional analysis will no longer be required for the routine analysis of such samples and GC × GC with FID is sufficient for quantitative analysis of the same oil. Shellie and co-workers [88] also made a critical comparison of GC-MS/FID results with GC × GC by comparison of nine different lavender oil produced in Australia. This qualitative and quantitative analysis revealed that GC × GC provided higher resolution and higher sensitivity compared to conventional GC.

The establishment or purchase cost of a TOFMS is a limiting factor for many analytical laboratories, however quadrupole MS (qMS) are prevalent. The first GC × GC with qMS for the analysis of plant extracts was described by Shellie and Marriott in 2003 [89]. To achieve this high resolution separation the authors reduced the mass scan range to 228 u to obtain a fast acquisition rate of 20 Hz and used a low pressure second dimension GC separation by using a wide bore second dimension column. The analysis used a non-polar × polar column combination and successfully identified 61 compounds from the *Pelargonium graveolens* essential oil with high quality spectra. Moreover the authors underlined that once performed the qualitative analysis then FID is sufficient for the quantitative analysis of same essential oils.

Later Tranchida and co-workers [90] reported a rapid-scanning qMS (20 spectra/s) for the reliable identification of a fresh and aged tea tree essential oils. This work consisted of a non-polar × polar column combination with a dual-stage loop type modulator able to separate 130 and 180 compounds from fresh and aged tea tree samples respectively. The authors affirmed that this three-dimensional enhanced-

resolution methodology enabled full separation of the samples analysed and noted that the differences of the essential oil constituents in both fresh and aged samples. Furthermore, they also showed the quantitative and qualitative improvements over convectional GC-MS analysis.

A comparative study of 13 pepper and peppercorn samples from different geographical origin contain more than 300 compounds using GC × GC-FID, GC × GC-qMS and GC × GC –TOFMS has been reported. The authors stated that *I* information from GC × GC-FID and mass spectral information from GC × GC-MS can be used for quantitative and qualitative analysis of plant extracts since both chromatograms were similar. In addition, they highlighted the benefits of qMS in plant extract analyses for very good peak identification assignments even though it has slow acquisition rate compared to TOFMS. In contrast the authors also noticed that the TOFMS allowed the identification of five times more components than qMS due to its capability of high acquisition rate, sensitivity and deconvolution, which constituted important attributes for hyphenation with GC × GC [91].

1. 4. Different approaches of high-resolution MDGC

The analyses of complex mixtures using multidimensional approaches have revealed that many samples are more complex than researchers realised. To separate all these individual compounds present in these samples are still a challenge for separation scientists.

A comprehensive GC × GC analysis of air sample showed that more than 5,000 compounds present at the low parts-per-trillion level and this inspired Ledford and co-workers [92] to develop a comprehensive three-dimensional gas chromatography (GC^3). This system was developed in a GC × GC instrument from Zoex Corporation,

modified through two staggered thermal modulators with three different stationary phase columns very short in length. A standard mixture comprising dodecane, tridecane, and tetradecane was separated with 120 min analysis time. The complexity of the hardware and software requirements limited application of this technique for real samples. In addition, the authors also noticed 33% of peak capacity loss in GC^3 compared to GC × GC.

In order to enhance resolution and limitations of previous reported GC^3 [92], Synovec's research group developed an alternate comprehensive GC^3 using two high-speed six-port diaphragm valves as the interfaces between three, in-series capillary columns housed in a gas chromatograph with FID detector [93]. The three capillary columns and dimensions were 5% diphenyl dimethyl polysiloxane (25 m × 0.53 mm i.d. × 5 μm d_f), trifluoropropylmethyl polysiloxane (5 m × 0.25 mm i.d. × 1 μm d_f) and polyethyleneglycol (1 m × 0.10 mm i.d. × 0.10 μm d_f) respectively. This instrument configuration provided separation on three different stationary phases in a single run. A standard mixture consisting of 26 individual compounds from nine different functional classes was used for calculating three retention times for single analytes and a chemometric parallel factor method used for quantitative analysis. Later the same research group used GC^3 for the analysis of diesel sample [94] with an ionic liquid column as second dimension column. The authors noticed the potential benefits of this three-dimensional comprehensive GC × GC system in terms of resolution and peak capacity. Furthermore the authors had intention to couple their novel approach with TOFMS and recommended to use thermally modulated system in order to enhance sensitivity and peak capacity since valve-based system transfers only a portion of the analyte [94].

More recently Sciarrone and co-workers [95] proposed a three-dimensional preparative gas chromatography method consisting of three GC systems with three Deans switch transfers and three FID detectors between three different capillary columns (first dimension : 5% diphenyl 95% dimethyl polysiloxane, 30 m × 0.53 mm i.d. × 5.0 μm d_f; second dimension: polyethylene glycol, 30 m × 0.53 mm i.d. × 2.0 μm d_f and third dimension was a custom made ionic liquid column (SLB-IL59), 30 m × 0.53 mm i.d. × 0.85μm d_f capillary column) for the analysis of wampee essential oil. The authors developed such system for both reliable identification and isolation of pure solutes from a complex mixtures, which are often hindered by a non-sufficient chromatographic separation [95]. Later the authors confirmed the unknown compound isolated from wampee essential oil with NMR, FTIR and MS information [95].

Maikhunthod and co-workers [96] proposed a system with switchable GC × GC and heart-cutting MDGC for the analysis of complex mixtures. This system consists of two different stationary phase capillary columns with a Deans switch, LMCS and two FID detectors. The primary column was 5% phenyl polysilphenylene-siloxane (30 m × 0.25 mm i.d. × 0.25 μm d_f) which was connected to a Deans switch that split the flow into two second dimension column with the same stationary phase but different physical dimensions. A short polyethylene glycol coated column (0.786 m × 0.1 mm i.d. × 0.1μm d_f) used for the GC × GC separation and a long polyethylene glycol coated column (30 m × 0.25 mm i.d. × 0.25 μm d_f) used for performing heart-cutting MDGC by switching the flow as desired. Such instrumental configuration provided additional separation power in complex mixtures where GC × GC fails to provide adequate separation power when complex analysis was performed. This is clearly shown by the authors for the analysis of fairly complex lavender essential oil.

Mitrevski and Marriott [97] proposed a novel hybrid GC × GC - MDGC for complex mixtures separation similar to Maikhunthod's [96] system. This system consists of three different stationary phases (first column polyethylene glycol: 30 m × 0.32 mm i.d. × 0.5 μm d_f; second column 5% diphenyl 95% dimethyl polysiloxane: 5 m × 0.15 mm i.d. × 0.15 μm d_f and third column 50% diphenyl 50% dimethyl polysiloxane: 20 m × 0.18 mm i.d. × 0.18 μm d_f) and two FID detectors. The first and second column connected through a longitudinal modulated cryogenic system and the end of second column connected with a Deans switch transfer. The Deans switch connected with one FID detector through a restrictor and a third column that terminates to the second FID detector. The first and second column provided a typical GC × GC data set and transfer the interested region to a third column by heart-cutting approach provided an additional separation power in single analysis for complex mixtures. The authors successfully utilised this approach for the analysis of stressed fuel oil and coffee volatiles.

Some researchers obtained high-resolution separations using a dual-secondary column comprehensive GC × 2GC [98-100]. A schematic representation of dual-secondary column comprehensive GC × 2GC shown in **Figure 1.5**. The concept first introduced Seeley's research group [98] in 2001 for the analysis of 130 volatile organic compounds containing different functional classes using a differential flow modulator made from high-speed six-port diaphragm valve. In order to achieve this instrumental

configuration the authors split the primary column effluent to two secondary columns instantly after the modulation process using a Tee-piece flow splitter. The primary column was 6% cyanopropylphenyl dimethyl polysiloxane (15 m × 0.25 mm i.d. × 1.4 μm d_f) and the two secondary columns were polyethylene glycol (5 m × 0.25 mm i.d. × 0.25 μm d_f) and 50% trifluoropropylmethyl polysiloxane (5 m × 0.25 mm i.d. ×

0.5 μm d_f) respectively connected with two FID detectors. This kind of instrumentation provided two sets of GC × GC chromatogram from two detectors with three retention times for single analytes (one primary and two secondary retention times). The authors outlined clearly the complementary nature of these three unique stationary phases for the analysis of volatile organic compounds and later in air and exhaled breath [98,100].

Similar version of GC × 2GC developed by Bieri and Marriott [99] using a LMCS instead of using differential flow modulator for the analysis of 24 suspected allergens used as fragrance ingredients. This study used a polyethylene glycol (30 m × 0.25 mm i.d. × 0.25 μm d_f) in the first dimension and 5% diphenyl 95% dimethyl polysiloxane (0.95 m × 0.10 mm i.d. × 0.1 μm d_f) and 14% cyanopropylphenyl dimethyl polysiloxane (0.95 m × 0.10 mm i.d. × 0.1 μm d_f) in the second dimensions. The authors emphasised the usefulness of *I* obtained from different stationary phases for compound identification in a complex mixture. The aforementioned GC × 2GC approach not only provided extra separation power but also provided three *I* values for single analytes from a single run, which is highly beneficial.

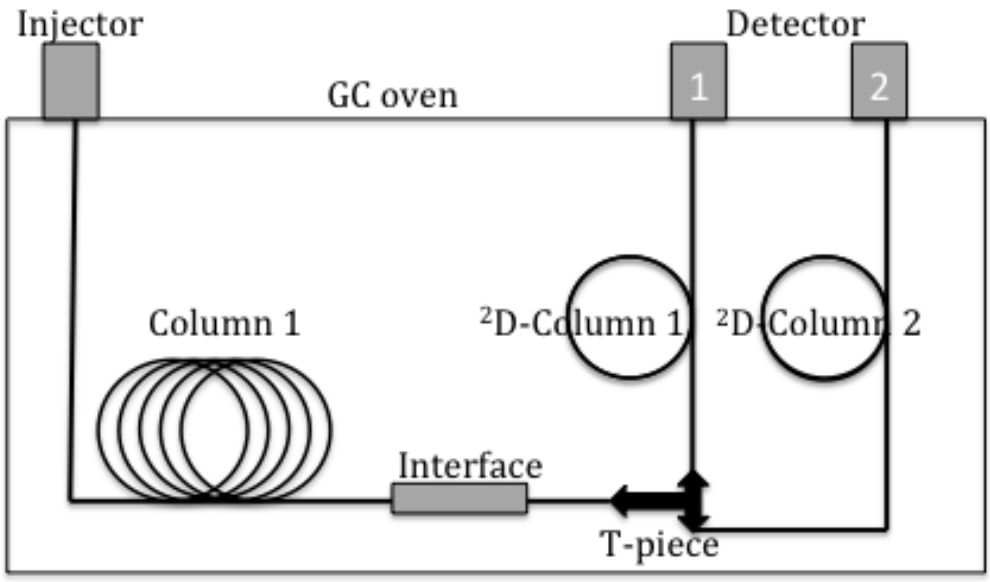

Figure 1.5. Schematic diagram of dual-secondary column comprehensive GC × 2GC instrumentation (adapted from references [98,99]). The T-piece split the modulated effluent coming from first-dimension column to the two second-dimension columns that ultimately ends with separate detectors.

In 2005 Adahchour and co-workers [101] introduced a twin GC × GC (2GC × 2GC) system using one four-jet two-stage modulation to simultaneously modulate both column sets. In 2GC × 2GC, the primary flow split into two first-dimension columns and then transferred into two second-dimensional columns configured in parallel in a single GC-oven, and connected with two FID detectors. The authors used a polar × non-polar (polyethylene glycol × 35% phenyl dimethyl polysiloxane) and non-polar × polar (dimethyl polysiloxane × polyethylene glycol) column set to initially analyse standard mixtures and then applied the set up for the analysis of fatty acids methyl esters (FAME's) and marine sediment extract. The authors emphasized the benefits of two independent GC × GC chromatogram from a single analysis, four retention times information for single analytes and complementary selectivity of four separation columns compared to their previous single GC × GC analysis [102].

A general comparison in terms of resolution, peak capacity, flexibility, complexity, number of detectors required,total analysis time and cost of all aforementioned approaches in GC is shown in **Table 1.1**. The instrumentation and methodologies of each mode of GC differ in many aspects. There are distinct pros and cons associated with each mode. GC × GC has unique advantages over the other high-resolution GC approaches in terms of resolution, peak capacity and analysis time. The cost of GC × GC analysis is high when it employs a cryogenic modulator, but it can be reduced by using alternatives such as pneumatic modulators or cooling modulators which do not require a cryogen, for instance the latest Leco modulator with chilled-cooled air and Zoex Thermal modulator with cooled loop modulation eliminate the use of cryogenic liquid. An addition of a third column in GC × GC brings more resolution in complex mixture analysis but the complexity, flexibility and analysis time needs trained experts to perform analysis.

Table 1.1. Comparison of different modes of GC system in terms of their features.

Features	Peak capacity	Resolution	Complexity	Flexibility	Number of detectors	Analysis time	Cost
IDGC	Moderate	Moderate	Very low	Very high	One	High	Low
Dual Channel GC	Moderate	High	Low	High	Two	High	Low
Heart-cutting MDGC	High	High	Very high	Moderate	Two/one	Very high	Moderate
GC × GC	Very high	Very high	High	Moderate	One	Moderate	High
GC^3	Very high	Very high	Very high	Very low	Three/Two/one	Very high	Very high
GC × 2GC	Very high	Very high	Very high	Low	Two	Moderate	High
GC × GC-MDCG	Very high	Very high	Very high	Very low	Two	High	Very high
2GC × 2GC	Very high	Very high	Very high	Low	Two	Moderate	High

1. 5. Scope of thesis

This thesis mainly focuses on developing a range of high-resolution separation techniques for plant extracts analysis. The work has a strong exploitation of capillary columns in terms of resolution, selectivity and analysis time of the separation in either one-dimensional GC or GC × GC approaches. The innovative high resolution GC separation approaches developed here are not limited to plant extracts analysis but can also be used for other complex mixtures and are easily coupled to MS.

This study initially explores the commercially available H_2 gas generator outlet pressure (100 psi) to obtain the best efficiency and speed within single dimensional GC separation using 20 m and 40 m narrow bore columns. The best trade-off between "efficiency and speed" for the analysis of *Tasmannia lanceolata* essential oil is studied under efficiency optimised flow or speed optimised flow conditions.

A series of novel GC approaches are explored utilising a new contra-directional modulation approached introduced in this thesis.

1. First the concept of dual-secondary column comprehensive GC (GC × 2GC) using a single detector system is explored. A new contra-directional thermal modulation regime is introduced for GC × 2GC using a quad-jet dual stage thermal modulator. This type of instrumental configuration provides a similar separation compared to previous GC × 2GC approach that relied on two detectors, but itself only requires one injector and one detector.

2. Further utilisation of the contra-directional thermal modulation mechanism helps to develop multiplexed dual-primary column comprehensive GC (2GC × GC). Instead of using two short second-dimension columns in GC × 2GC, two

conventional 30 m long columns are used in the first dimension to provide two sets of GC × GC chromatograms, with three retention times, from a single detector system. This instrumentation used for the analysis of plant extracts to provide two complementary chromatograms that facilitate correct compound identifications. The approach is compared with GC × 2GC and normal GC × GC analysis proved the benefits in the case of plant extract analysis.

3. Later, approach 2 was coupled to MS for reliable identification of plant extracts.

4. The development of multiplexed 2GC system with a single detector is also discussed. This study once again exploited the contra-directional thermal modulation for achieving 2GC-MS/FID systems. The current 2GC system requires single detector to provide two *I* from non-polar and polar columns will ease the analysis and high sample throughput can be attained.

5. Finally, this thesis developed a most powerful multidimensional instrumental configuration using the contra-directional modulation regime. The approach is called "2GC × 2GC, or 2(GC × GC)". The potential benefits of such an enabling instrumentation is shown by analysing plant extracts. From this instrumentation two sets of GC × GC chromatogram can be achieved with four retention times for each analyte from a single analysis.

Chapter 2

Use of long, narrow-bore capillary gas chromatography columns for detailed characterisation of *Tasmannia lanceolata* extract

2. 1. Introduction

Essential oils, concretes, and absolutes are complex mixtures that require a high peak capacity for satisfactory chromatographic separation [39]. *Tasmannia lanceolata* (Poir.) A.C. Smith, commonly called Mountain Pepper, is a bush food plant from the *Winteraceae* family. There is increasing interest in using *T. lanceolata* extracts as natural flavour ingredients, however detailed qualitative understanding of the plant extract will be required for regulatory approval. "GRAS" is an acronym for the phrase Generally Recognized as Safe [103]. According to the United States Food and Drug Administration, the use of a food substance may be GRAS either through scientific procedures or, for a substance used in food before 1958, through experience based on common use in food [103]. To date only a small number of reports concerning the chemical constitution of *T. lanceolata* extracts are available in the open literature, with fewer than seventy components hitherto reported in the volatile fraction [91,104-107]. While some reports [108-111] focus on determination of the major constituent polygodial [CAS 6754-20-7], the limited qualitative information may also be ascribed to insufficient peak capacity to provide adequate chromatographic resolution for more detailed characterisation.

A universally applicable approach for high-resolution GC analysis is to employ long capillary columns. To this end, several studies have focused on the use of long capillary columns for the analysis of complex mixtures. Notably Desty, Goldup and Swanton [112] used a 263 m × 0.14 mm internal diameter (i.d.) column for 3.5 h analysis of a crude petroleum sample. Berger utilised a 450 m × 0.20 mm i.d. capillary column for gasoline analysis [10]. Separation of 950 peaks was possible in a 650 min temperature-programmed analysis. More recently, Mydlová-

Memersheimerová *et al.* [13] reported the separation of 195 out of 209 polychlorinated biphenyl congeners using an 80 m × 0.10 mm i.d. capillary column.

High peak capacity comes at a cost of increased analysis time. Giddings introduced expressions for the maximum number of theoretical plates which can be attained in GC analysis using packed columns [113]. Desty *et al* [112] attempted to express the column performance as the ratio of the number of effective theoretical plates to the analysis time and defined the optimum practical gas velocity as the velocity for the maximum of N/t_R. The best trade-off between efficiency and speed continues to be an active research interest in the liquid chromatography literature using kinetic performance characterisation [114-118]. Hinshaw [119] discussed the various aspects of GC theory with practical examples in order to obtain the best trade-off between efficiency and speed. The author suggested operating the column above optimum linear velocities, reducing the total analysis time while not reducing the efficiency by much. Blumberg [120] expressed the column performance in terms of gas flow rate and introduced efficiency-optimised gas flow rate and speed-optimised gas flow rate conditions. The efficiency-optimised gas flow rate optimise with a fixed column length to generate maximum efficiency, while speed-optimised gas flow rate optimise with a fixed efficiency by varying column length and gas flow rate, and, hence, the shortest analysis time for any fixed efficiency.

It is well known that decreasing column length, reducing internal diameter, selecting hydrogen as carrier gas, using faster temperature programing, etc. lead to increased analysis speed in GC. Blumberg and Klee [121] proposed an optimal heating rate for high pressure drop separation is about 10 ^{o}C per void time. The most widely used and easily accessible narrow-bore columns appropriate for complex mixture separation are those 0.10 mm internal diameter [122,123].

The present study investigated the best trade-off between speed and efficiency using a narrow bore column with hydrogen carrier gas that generated from a commercially available gas generator. The maximum outlet pressure of this generator (100 psi) allows some variation of column length to optimise speed and efficiency. A 20 m and 40 m coupled-column was selected and operated either efficiency-optimised gas flow rate or speed-optimised gas flow rate conditions for detailed characterisation of *T. lanceolata* plant extract using GC-MS system. The experiment described in this chapter helped the Tasmanian essential oil industry to obtain a detailed characterisation of *T. lanceolata* sample components for regulatory approval.

2. 2. Experimental

Samples

Viscous, dark green *T. lanceolata* concrete was provided by Essential Oils of Tasmania (Kingston, Australia). The concrete was diluted 10% v/v in dichloromethane (Sigma-Aldrich, Castle Hill, Australia). A 0.001% v/v C_8-C_{20} *n*-alkane standard mixture (Sigma-Aldrich) for determination of retention indices and separation number was prepared in dichloromethane.

Instrumentation

All analyses were performed on a Shimadzu QP2010 GC-MS equipped with an AOC-20i auto-injector, split/splitless injector, and quadrupole mass spectrometer (Shimadzu Oceania, Rydalmere, Australia). Throughout the study, 20 m × 0.10 mm i.d. capillary columns coated with a thin layer (0.10 µm d_f) of polyethylene glycol stationary phase and 5% diphenyl 95% dimethyl polysiloxane phase (Rtx-Wax and Rtx-5; Restek, Bellefonte, PA, USA) were used. SilTite unions (SGE Analytical

Science, Ringwood, Australia) were used to join individual 20 m columns in series to make 40 m columns. All columns shared the same manufacturer batch number. Hydrogen carrier gas was generated using a Parker Balston Hydrogen Generator (Leco, Castle Hill, Australia). A 1 µL split injection at 230 ^{o}C was employed for all separations with a split ratio of 100:1. The efficiency optimised flow condition of 20 m columns were operated at 40.16 psi with an initial flow rate of 0.5 mL/min and the temperature is programmed initially 45 ^{o}C (hold 2.4 min) and then increased to 240 ^{o}C at a temperature program rate of 7.5 ^{o}C/min. The 40 m columns were operated at 62.53 psi with an initial flow rate of 0.5 mL/min and the temperature is programmed initially 45 ^{o}C (hold 6.45 min) and then increased to 240 ^{o}C at a temperature program rate of 2.8 ^{o}C/min. Speed optimised flow rate condition of 40 m columns were operated at 100 psi with an initial flow rate of 1.1 mL/min and the temperature is programmed initially 45 ^{o}C (hold 4.25 min) and then increased to 240 ^{o}C at a temperature program rate of 4.25 ^{o}C/min. The MS scan parameters were: mass range 35-350 m/z, scan intervals 0.1s, scan speed 5000 amu/s and detector voltage was relative to the tuning result which was 2.7 kV and less. The MS ion source and transfer line temperature was 200 ^{o}C and 250 ^{o}C respectively.

Data analysis

Peak identification was performed using Shimadzu GC-MS Solution, using the library search feature. Acquired spectra were compared with the Flavour and Fragrance Natural and Synthetic Compounds (FFNSC) GC-MS library. The interactive retention index filter was used to eliminate false positive matches by applying a search window of ± 10 Linear Retention Index (LRI) units.

2. 3. Results and Discussion

2. 3. 1 Performance of 20 m and 40 m capillary columns

In this investigation, a series of analyses were performed using off-the-shelf, 20 m columns to provide benchmark performance metrics for comparison with coupled-column ensembles. The measured estimated peak capacity determined by summing the separation number between *n*-alkane homologues, from *n*-octane to *n*-eicosane for the 20 m and 40 m coupled-columns is given in **Table 2.1**.

Under efficiency-optimised conditions, 20 m columns produced peak capacities of 671 ± 5 (polyethylene glycol) and 684 ± 3 (5% diphenyl 95% dimethyl polysiloxane). The average separation number between neighbouring *n*-alkane homologues was 56 and 57 respectively. The analysis time, based on adjusted retention time of the last peak eluted was less than 25 min for both columns. Also under efficiency-optimised conditions, the average peak capacity of the 40 m coupled-columns was 1033 ± 36 and 1067 ± 26 with analysis time under 66 min.

The long, narrow-bore columns used in the present study predictably showed significantly better performance than the 25 m × 0.25 mm i.d. column used by Bicchi *et al* [124] in terms of efficiency and analysis time. These authors reported that the available peak capacity of a 25 m × 0.25 mm i.d. × 0.30 µm polar wax column was 266.6. A separation number between two homologue pairs in the Grob test mixture was 31.7 with an analysis time more than 50 min.

The points discussed above represent the extremes of the triangle of compromise (**Figure 2.1**), which is a useful cartoon to illustrate the need to trade-off desirable outcomes to maximise other outcomes. Bicchi *et al.* [124] elected to sacrifice

Table 2.1. Average separation number and 95% confidence interval determined from triplicate injection of C_8-C_{20} normal alkanes. Columns marked with an asterisk were operated using efficiency-optimised conditions. All others were operated using speed-optimised conditions.

Column	separation number (SN)													$t'_{R\,(C20)}$ (min)
	8-9	9-10	10-11	11-12	12-13	13-14	14-15	15-16	16-17	17-18	18-19	19-20	8-20	
20 m Rtx-Wax*	53±6	61±3	72±1	80±4	68±4	60±1	57±2	53±3	46±2	42±1	40±1	39±5	671 ± 5	17.1±0.001
20 m Rtx-5*	65±9	73±3	74±3	69±2	64±2	61±2	55±2	52±2	48±1	42±4	43±3	38±1	684 ± 3	24.6±0.025
40 m Rtx-Wax*	90±2	113±9	121±3	120±8	110±8	96±7	88±3	69±8	65±8	64±3	52±5	45±4	1033 ± 36	49.3±0.009
40 m Rtx-5*	108±2	116±3	124±8	109±10	98±4	91±3	84±4	77±5	73±4	66±1	61±3	60±4	1067 ± 26	65.5±0.003
40 m Rtx-Wax	90±2	112±4	118±6	109±5	101±2	89±2	76±3	68±7	63±9	62±4	54±5	41±1	983 ± 12	32.3±0.002
40 m Rtx-5	106±3	113±4	111±2	104±3	97±4	88±3	78±1	72±4	68±3	62±2	57±3	54±7	1010 ± 17	43.3±0.016

efficiency to generate speed. The efficiency-optimised approach does not consider speed as an indicator of suitability, and the increased efficiency achieved by using a long column is understandably counterbalanced by the associated increase in analysis time. The speed optimised flow lies somewhere along the side of the triangle, between the speed and efficiency vertices. For the 40 m coupled-columns the average peak capacity value was on the order of 1,000 with analysis time less than 44 min. Comparing efficiency-optimised results, increasing the column length offered an improvement in peak capacity by a factor of 1.55, with analysis time increased by a factor of 2.6. The results of speed optimised method development give 1.47 times better peak capacity (than the 20 m column) but only increased analysis time by a factor of 1.8. In other words the speed-optimised separations are 30% faster than the efficiency optimised separations using the same column length, but they are only 5% worse in terms of performance. This is a very satisfactory practical compromise. The high peak capacity separations from the present study compare favourably to the recent study of Mydlová-Memersheimerová *et al*. [13] in which the available peak capacity of an 5% diphenyl 95% dimethyl polysiloxane 80 m × 0.10 mm i.d. column was calculated theoretically to be 1198 and practically measured to be 724. To make a fair comparison with the study of Mydlova-Memersheimerová *et.al* [13] we chose to calculate a peak capacity value using similar minimum and maximum retention indices. As PCB numbers 1 and 61 correspond to retention indices of approximately 1500 and 2010 respectively [125] we calculated the peak capacity for our study between C_{15} and C_{20} *n*-alkanes to compare with the value of 305 calculated for the analysis of PCBs 1 to 61 in ref [13]. Calculations of the peak capacity for this study using the 5% diphenyl 95% dimethyl polysiloxane 40 m long narrow bore column under efficiency optimised flow conditions and speed optimised flow conditions gave

values of 337 and 313 respectively. Although the performance of 20 m and 40 m narrow bore capillary column provided a satisfactory peak capacity with an acceptable analysis time, the disadvantage associated with such column is the low sample capacity. The upper mass limit per peak drops off quickly after reducing the column inner diameter, but separation and efficiency are maintained [126]. In many complex mixtures particularly in essential oil, the major components may be present in concentrations that are orders of magnitude greater than minor components. However, analysis of both dilute and concentrate samples will most likely become requirement.

2.3.2 Analysis of *Tasmannia lanceolata* extract

First, separations were performed using the 20 m and 40 m polar columns for comparison of efficiency optimised and speed optimised conditions for the analysis of *T. Lanceolata*, (**Figure 2.2**). Based on the data collected 84 peaks were apparently resolved using the polyethylene glycol column under efficiency optimised conditions. More compounds were separated when increasing the column length to 40 m for the same sample; 116 peaks were apparently resolved under efficiency-optimised conditions (analysis time of 65 min). The same number of peaks was resolved under speed-optimised conditions with a total analysis time of 43 min. The detection of highly retained low concentrated compounds using the 40 m polar column was affected due to the column bleeding at high temperature program rates (the upper operation temperature limitation of polyethylene glycol column used in this study was 240 ^{o}C recommended by the manufacturer) shown in Figure 2.2b and Figure 2.2c. It is recommended using a highly thermally stable (low bleed) column will be avoided this issue. Nevertheless, the availability of retention indices information for those columns at least limits them to use qualitative analysis of plant extracts.

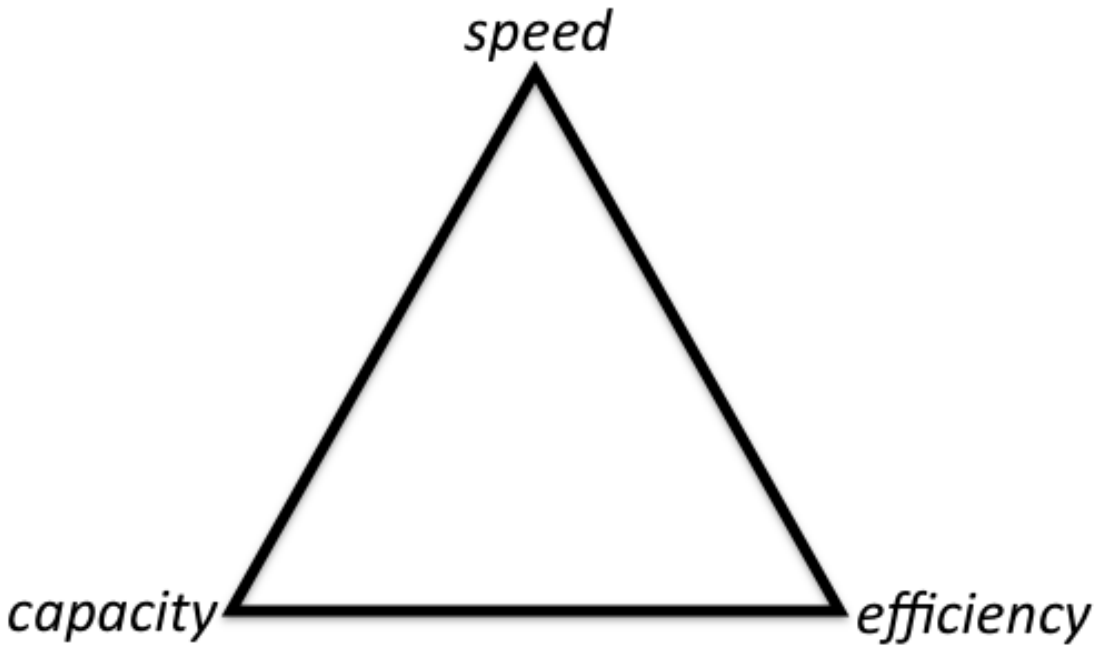

Figure 2.1. The "triangle of compromise". The vertices of the triangle represent the maximum possible speed, efficiency, and sample capacity. In order to maximise one of these, the others have to be compromised.

Using the 20 m non-polar column **(Figure 2.3)** 102 peaks were resolved under efficiency-optimised conditions. 135 peaks were resolved using the 40 m column under the efficiency-optimised condition in 65 min, but using the speed-optimised conditions allowed the same number of peaks to be resolved in only 43 min.

The qualitative analysis of *T.lanceolata* extract was carried out using the 5% diphenyl 95% dimethyl polysiloxane 40 m non-polar column. The number of peaks identified in the volatile fraction of the oil is shown in **Table 2.2**. The robust identification is made by comparing mass spectra and LRI with a reliable MS library and using appropriate search filters such as FFNSC1.3. Polygodial is not available on commercial libraries, so this compound was identified using an internal library and knowledge this component is the major constituent of the oil. Out of 135 detected volatile components of the oil, 84 peaks were identified. This is the first report of such large number of volatile fraction of the *T. lanceolata* oil constituents. Cardeal *et al*. employed comprehensive two-dimensional gas chromatography (GC × GC) coupled to mass spectrometry (MS) for qualitative analysis of dry *T. lanceolata* pepper berries, and identified sixty-nine volatile compounds [91]. Previously only a limited number of papers have been published detailing the chemical constituents of *T. lanceolata* essential oil with approximately 40 peaks characterised using GC [104,127-129]. From Figure 2.3 and Table 2.2 it can be seen that no reduction in the number of observed peaks using the 40 m non-polar column operated under either efficiency optimised flow or speed optimised flow conditions. This result is very promising for a trade-off between efficiency and analysis time. Moreover, the average percentage of MS library hits for identified component was more than 90% and retention indices matched literature values within ± 10 units.

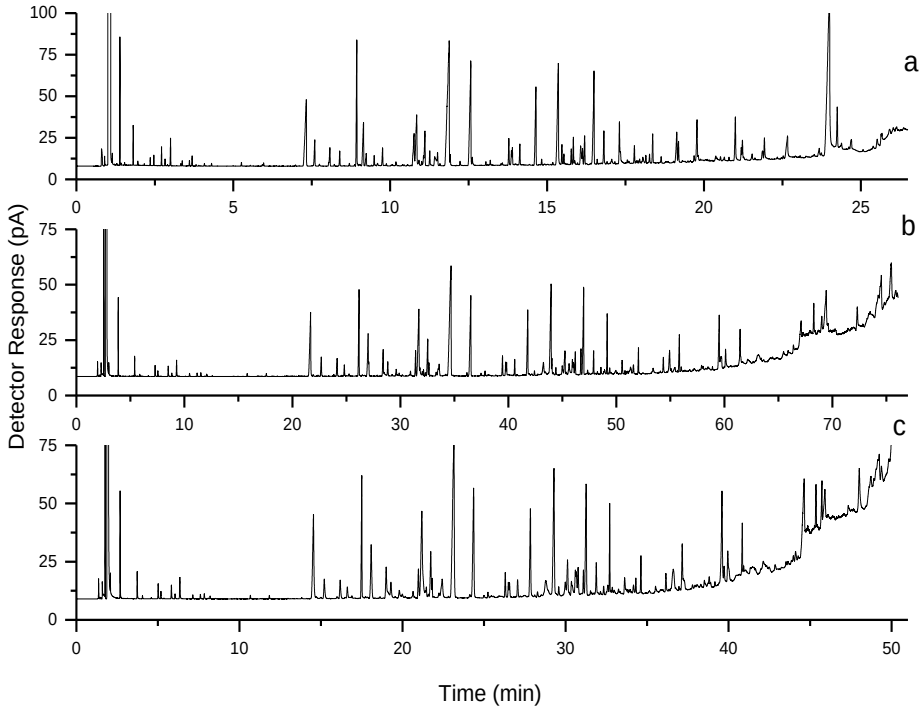

Figure 2.2. Separation of *T. lanceolata* extract using Rtx-Wax; polyethylene glycol column a) 20 m x 0.10 mm i.d. b) 40 m × 0.10 mm i.d operated at efficiency-optimised condition and c) 40 m × 0.10 mm i.d operated under speed optimised condition.

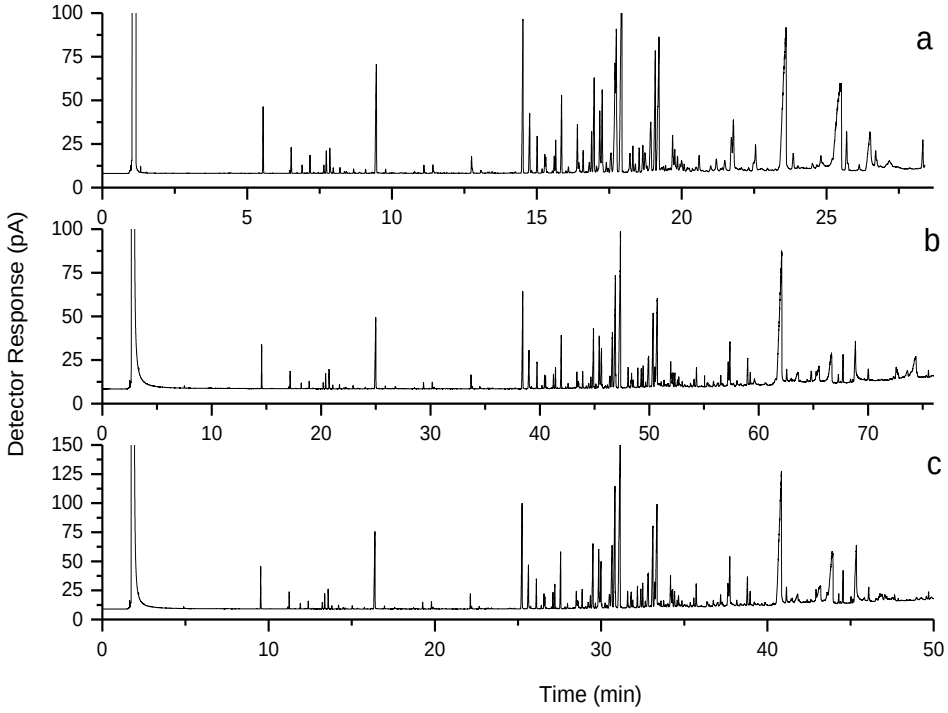

Figure 2.3. Separation of *T. lanceolata* extract using Rtx-5; 5% diphenyl 95% dimethyl polysiloxane a) 20 m x 0.10 mm i.d. b) 40 m × 0.10 mm i.d operated under efficiency-optimised condition and c) 40 m × 0.10 mm i.d operated under speed optimised condition.

Table 2.2. Peak identification after GC-MS analysis and comparison of observed and library linear retention indices.

LRI	Compound	MW	Formula	Library Match	LRI (Library)	Δ LRI
932	α-pinene	136	C10 H16	97	933	1
973	sabinene	136	C10 H16	95	972	-1
976	β-pinene	136	C10 H16	95	978	2
992	myrcene	136	C10 H16	96	991	-1
1011	δ-3-carene	136	C10 H16	94	1009	-2
1025	p-cymene	134	C10 H14	98	1025	0
1029	limonene	136	C10 H16	92	1030	1
1034	eucalyptol	154	C10 H18 O	97	1032	-2
1038	(Z)-β-ocimene	136	C10 H16	93	1035	-3
1049	(E)-β-ocimene	136	C10 H16	91	1046	-3
1089	terpinolene	136	C10 H16	95	1086	-3
1105	linalool	154	C10 H18 O	90	1101	-4
1120	3-methyl-,3-butenyl-3-methyl butyrate	170	C10 H18 O2	95	1114	-6
1196	α-terpineol	154	C10 H18 O	87	1198	2
1261	piperitone	152	C10 H16 O	93	1267	6
1290	bornyl acetate	196	C12 H20 O2	92	1285	-5
1338	δ-elemene	204	C15 H24	87	1335	-3
1351	α-cubebene	204	C15 H24	97	1349	-2
1365	eugenol	164	C10 H12 O2	96	1357	-8
1377	α-copaene	204	C15 H24	98	1375	-2
1385	β-bourbonene	204	C15 H24	97	1382	-3
1391	β-cubebene	204	C15 H24	93	1392	1

1393	β-elemene	204	C15 H24	94	1390	-3
1404	italicene	204	C15 H24	96	1410	6
1407	(Z)-α-bergamotene	204	C15 H24	91	1416	9
1421	(E)-caryophyllene	204	C15 H24	96	1418	-3
1430	γ-elemene	204	C15 H24	87	1432	2
1433	calarene	204	C15 H24	94	1434	1
1437	(E)-α-bergamotene	204	C15 H24	96	1432	-5
1440	α-guaiene	204	C15 H24	95	1438	-2
1452	guia-6,9-diene	204	C15 H24	88	1444	-8
1455	α-humulene	204	C15 H24	95	1454	-1
1459	(E)-β-farnesene	204	C15 H24	95	1452	-7
1463	9-epi-(E)-caryophyllene	204	C15 H24	96	1464	1
1476	cadina-1(6),4-diene	204	C15 H24	93	1472	-4
1482	γ-curcumene	204	C15 H24	83	1482	0
1484	germacrene D	204	C15 H24	96	1480	-4
1485	α-curcumene	202	C15 H22	91	1480	-5
1488	β-selinene	204	C15 H24	97	1492	4
1497	viridiflorene	204	C15 H24	90	1491	-6
1499	bicyclogermacrene	204	C15 H24	91	1497	-2
1503	α-muurolene	204	C15 H24	98	1497	-6
1508	α-bulnesene	204	C15 H24	90	1505	-3
1512	β-bisabolene	204	C15 H24	92	1508	-4
1515	β-curcumene	204	C15 H24	94	1511	-4
1517	γ-cadinene	204	C15 H24	89	1512	-5
1519	β-sesquiphellandrene	204	C15 H24	83	1523	4
1522	δ-cadinene	204	C15 H24	91	1518	-4
1527	(E)-calamenene	202	C15 H22	95	1527	0

1536	1,2,3,4,4a,7-hexahydro-, 1,6-dimethyl-, 4-(1-methylethyl)-, (1alpha,4beta,4abeta)-naphthalene	204	C15 H24	96	1536	0
1556	germacrene B	204	C15 H24	90	1557	1
1561	dihydroisocaryophyllene epoxide	222	C15 H26 O	88	1565	4
1569	(E)-nerolidol	222	C15 H26 O	91	1561	-8
1573	ledol	222	C15 H26 O	94	1574	1
1578	spathulenol	220	C15 H24 O	85	1576	-2
1589	caryophyllene oxide	220	C15 H24 O	91	1587	-2
1606	guaiol	222	C15 H26 O	86	1603	-3
1611	tetradec-(9Z)-enal	210	C14 H26 O	83	1611	0
1629	epicubenol	222	C15 H26 O	87	1631	2
1632	γ-eudesmol	222	C15 H26 O	87	1632	0
1636	1,2,3,4,4a,7,8,8a-octahydro-, 2,4a,5,8a-tetramethyl-formate napthalen-1-ol	236	C15 H24 O2	80	1643	7
1649	1,2,3,4,4a,7,8,8a-octahydro-, 4-isopropyl-, 1,6-dimethyl-naphth-1-ol	222	C15 H26 O	90	1641	-8
1654	α-muurolol	222	C15 H26 O	90	1651	-3
1658	β-eudesmol	222	C15 H26 O	83	1656	-2
1662	cadina-4-en-10-ol	222	C15 H26 O	90	1659	-3
1675	bulnesol	222	C15 H26 O	91	1673	-2
1685	tetradeca-(9Z, 12E)-dien-1-ol	210	C14 H26 O	85	1676	-9
1699	caryophyllene acetate	264	C17 H28 O2	88	1701	2
1770	cedrenyl acetate	262	C17 H26 O2	88	1771	1

1773	drimenol	222	C15 H26 O	96	1769	-4
2012	polygodial	234	C15 H22 O2	N/A	N/A	N/A

2. 4. Conclusion

Based on the characterisation of kinetic performance a high-resolution capillary GC approach was developed that is applicable to the analysis of complex plant extracts. It is very significant to perform comprehensive characterisation of natural products intended as food ingredients. The approach developed here provides satisfactory separation of *T. lanceolata* extracts. Flame ionisation detection is a preferred technology for performing quantitative analysis, and the described approach is suitable for routine quality assurance. It is appealing that the speed-optimised separation is completed within 45 min. In contrast an efficiency-optimised separation requires more than 60 min, but only offers a marginal improvement in peak capacity. However, in the case of *T.lanceolata* extract it was found that there is no reduction in the number of observed peaks using the speed optimised conditions. Higher kinetic performance could be reached by using UHP H_2 cylinder and longer columns at higher pressure.

Chapter 3

Multiplexed dual second-dimension column comprehensive two-dimensional gas chromatography (GC × 2GC) using thermal modulation and contra-directional second-dimension columns

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3. 1. Introduction

In 2001 Seeley and co-workers introduced a differential flow modulation comprehensive two-dimensional gas chromatography (GC × GC) instrument configuration incorporating two second-dimension columns [98]. This GC × GC instrument configuration split the effluent leaving a differential flow modulator into two 5 m × 0.25 mm i.d. second-dimension columns. Effluent from each of the second-dimension columns was monitored by flame ionisation detection (FID). The resulting dual-secondary column comprehensive two-dimensional gas chromatography (GC × 2GC) technique produced a pair of two-dimensional chromatograms in a single run. When careful consideration is given to stationary phase selection, GC × 2GC provides superior resolution and qualitative information compared to GC × GC analysis. This was demonstrated in reference [98], which examined the use of a 6% cyanopropylphenyl, 94% dimethyl polysiloxane first-dimension column coupled to polyethylene glycol and trifluoropropylmethyl polysiloxane second-dimension columns. The same GC × 2GC system was used further for qualitative analysis of volatile organic compounds in exhaled breath, outdoor air, and chemical stockroom air [100]. Bieri and Marriott adapted the GC × 2GC approach later in an experiment designed to simultaneously generate three retention indices for volatile compounds [99]. The primary flow of the first-dimension column was equally diverted into two second-dimension columns using a three-way flow splitter positioned after a longitudinally modulated cryogenic system (LMCS). Simultaneous collection of three retention indices is highly advantageous because identification capabilities are close to those obtainable with mass spectrometric data when retention index information is available on different stationary phases [22].

Unfortunately each of the aforementioned GC × 2GC approaches necessitate use of a detector for each second-dimension column. While this is not generally problematic if an inexpensive detection approach, such as FID is employed, dual selective detectors may be prohibitively expensive for some users. GC × 2GC-MS has not been reported despite multiplexed HPLC-MS being well established. For instance, parallel liquid chromatography systems with mass spectrometric detection using a multiplex electrospray source for direct, sensitive determination of pharmaceuticals in plasma, such as that introduced by Mallett and co-workers [130] are widely used in the pharmaceutical industry for high-throughput screening.

In the present chapter, an enabling instrument configuration that generates dual GC × GC chromatograms in a single run without requiring multiple detectors is introduced. Demonstration is provided with a single FID and further work will couple the approach with mass spectrometry to explore the full potential of multiplexed GC × 2GC.

3. 2. Experimental

A test sample comprising a mixture of 34 volatile organic compounds (**Table 3.1**) was prepared in dichloromethane (~ 5%). All compounds were obtained from Sigma-Aldrich in purity exceeding 97%, where available.

All analyses were performed using a Leco GC × GC-FID instrument with LN2 Cooled Thermal Modulator (Leco Australia, Castle Hill, Australia). The chromatograph was equipped with split/splitless injector, operated in split mode throughout (20:1) and 230 ^{o}C. Hydrogen carrier gas was generated using a Parker Balston H2PEM-260 hydrogen generator and supplied at an initial head pressure of 69 kPa. The GC was operated in constant flow mode, providing a calculated flow rate

of approx. 1.7 mL/min. A 10 m × 0.25 mm i.d. first-dimension separation column with an Rtx-624 low to mid polarity 6% cyanopropylphenyl / 94% dimethyl polysiloxane stationary phase (1.4 μm film thickness) was employed in this study. The first-dimension column was connected to two second-dimension columns using a 3-port SilFlow connector (SGE Analytical Science, Ringwood, Australia). Second-dimension *Column 1* was a 1.0 m × 0.15 mm i.d. Rtx-Wax polar polyethylene glycol stationary phase column (0.15 μm film thickness). Second-dimension *Column 2* was a 1.0 m × 0.15 mm i.d. Rtx-200 mid polarity trifluoropropylmethyl polysiloxane stationary phase column (0.15 μm film thickness). Flow from the second-dimension columns was recombined using a deactivated universal Y- angled presstight connector (Restek, Bellefonte, USA) into a 0.5m × 0.25 mm i.d. deactivated fused silica transfer line that terminated at the FID (this adds 0.82 s in the second-dimension analysis time). All separation columns were from Restek (Bellefonte, USA). A temperature program of 40 – 150 ^{o}C (5 ^{o}C/min) was used throughout. The second dimension columns were installed contra-directionally in the GC × GC modulator. For convenience of column installation and operation, the auxiliary (second-dimension) column oven was removed from the GC × GC system. All three columns were heated using the main GC oven. Modulation timing parameters are described in Results and Discussion section. The modulator offset temperature was 15 ^{o}C. Effluent from each secondary column was monitored by a single flame-ionisation detector. The data acquisition rate and the operating temperature were 100 Hz and 250 ^{o}C respectively. Data were collected and summarized (retention times, peak areas) using Leco ChromaTOF software.

3. 3. Results and discussion

Multiplexed GC × 2GC is performed by employing contra-directional modulation – by installing the second-dimension columns contra-directionally in the GC × GC modulator (**Figure 3.1**). Contra-directional modulation (**Figure 3.2**) employs a conventional dual stage thermal modulator, and works according to the same principles [131], but utilises the principles of dual stage thermal modulation to further benefit. Consider a column configuration comprising only columns A and B. This system represents a conventional GC × GC setup. The second (top) stage of the dual-stage thermal modulator plays a primary role in focusing and releasing solute into the second dimension column (column B), while the first (bottom) stage of the modulator acts to prevent breakthrough [132]. Periodically cycling the hot and cold modulator gases leads to a GC × GC chromatogram. Next, consider a column configuration comprising columns A and C. Here the two modulation stages are inverted compared to a conventional GC × GC setup. The second (bottom) stage of the dual-stage thermal modulator plays a primary role in focusing and releasing solute into the second dimension column (column C), while the first (top) stage of the modulator acts to prevent breakthrough. The only difference between the two described configurations is the time (after injection) that a solute eluted from the first dimension column is injected into the second dimension column. These times differ because the two stages are never hot at the same time. Combined with modified modulation timing parameters, contra-directional column installation permits multiplexed GC × 2GC.

Modulator timing parameters are key to achieving multiplexed GC × 2GC. In the present investigation, symmetrical modulation timing is employed throughout. **Figure 3.3** illustrates a typical modulation-timing scheme for multiplexed GC × 2GC with

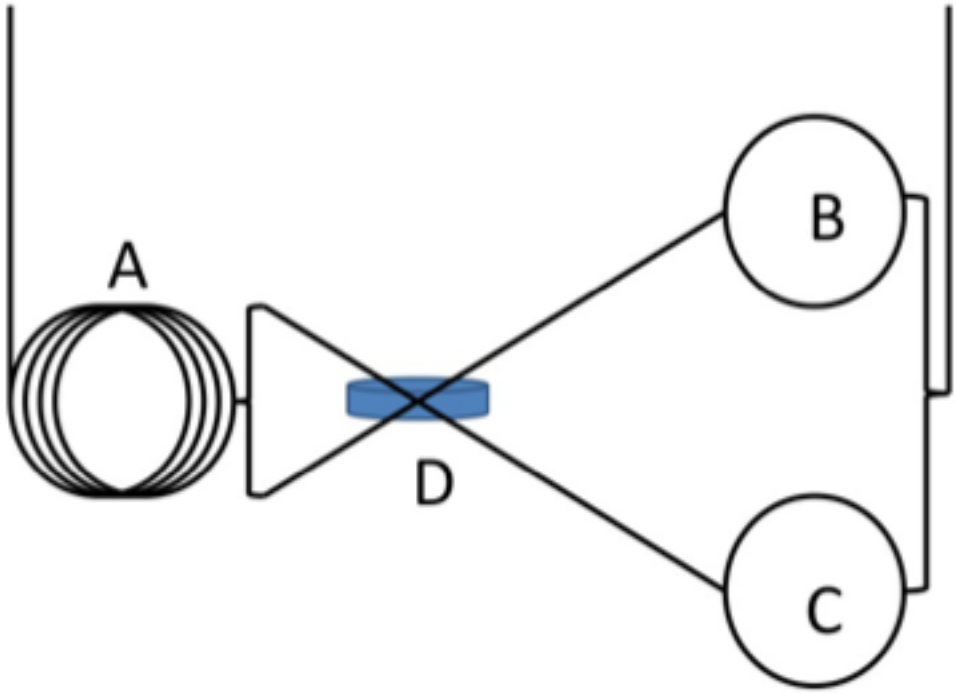

Figure 3.1. Illustration of the column configuration used for multiplexed GC × 2GC.

A, first-dimension column; B, second-dimension *Column 1*; C, second-dimension *Column 2*; D, dual stage thermal modulator

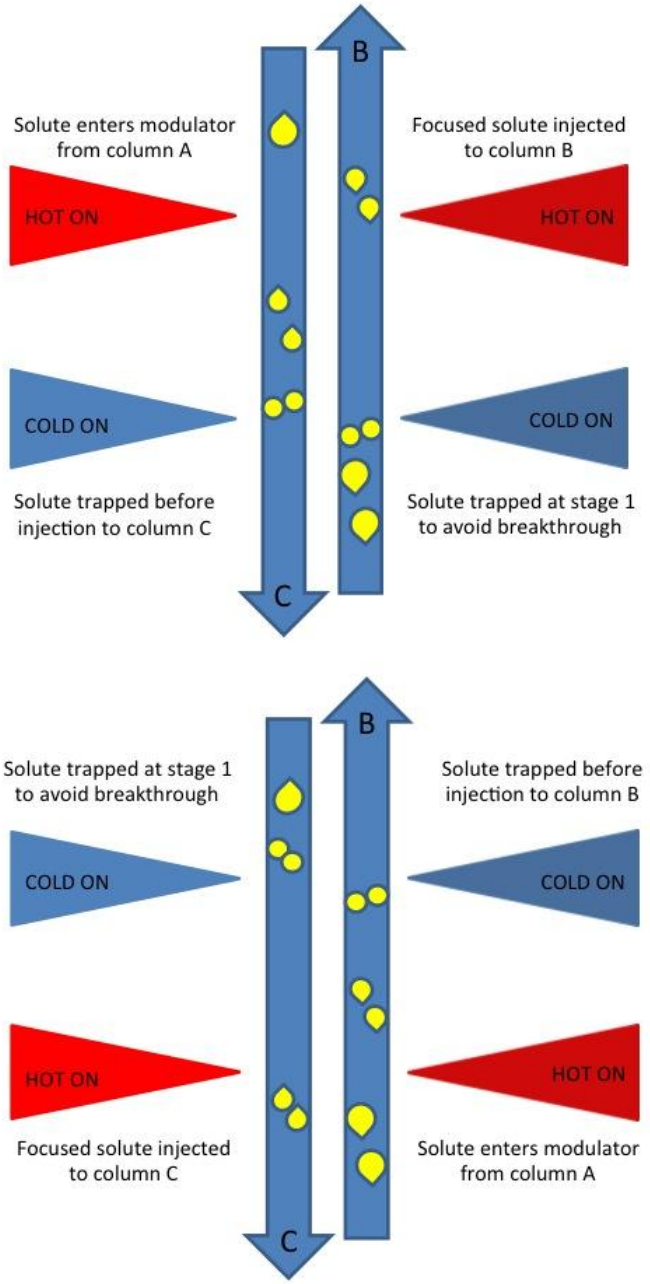

Figure 3.2. Illustration of contra-directional dual second-dimension columns installed in a dual-stage thermal modulator. See Figure 3.1 for a sketch of complete column connectivity.

contra-directional second- dimension columns. The grey lines indicate the hot jet timing parameters and black lines indicate the cold jet timing parameters of the dual stage modulator. Second-dimension *Column 1* is installed in the dual stage modulator in a conventional manner, namely from the bottom-up. The other second-dimension column is installed from the top-down. In this way, the direction of carrier gas flow through the second-dimensional columns in the modulator is *contra-directional*. Each of the second-dimension columns is continuously operated, but the injection into each second-dimension column is delayed by the total modulation period. The total modulation period is selected so that it provides excessive separation space for any of the second-dimension separations. The excess separation space provides vacant separation space for peaks eluting from the alternate second-dimension column to fill. In this way, once peaks are split into the two second-dimension columns, they are never re-mixed. Figure 3.3 shows that the upward and downward modulation cycles are identical, except they are out of phase by exactly half of the total modulation period.

Challenges with implementing this GC × 2GC approach include maintaining modulation ratio [73] in line with accepted guidelines, to ensure sufficient sampling to minimise modulation-induced loss first-dimension of resolution [71]. By nature of the contra-directional modulation approach, the modulation period must be double what is normally acceptable. In the present investigation, a 12 s modulation period (providing M_R of >1) is applied. The two-dimensional separation space required to fit the separated compounds from each second-dimension column is 6 s, and the vacant 6 s is utilised for the alternate second-dimension separation. Careful attention needs to be paid to avoid wrap-around because this would be highly detrimental to the integrity of the multiplexed GC × 2GC separation. Wrap-around has been avoided in the

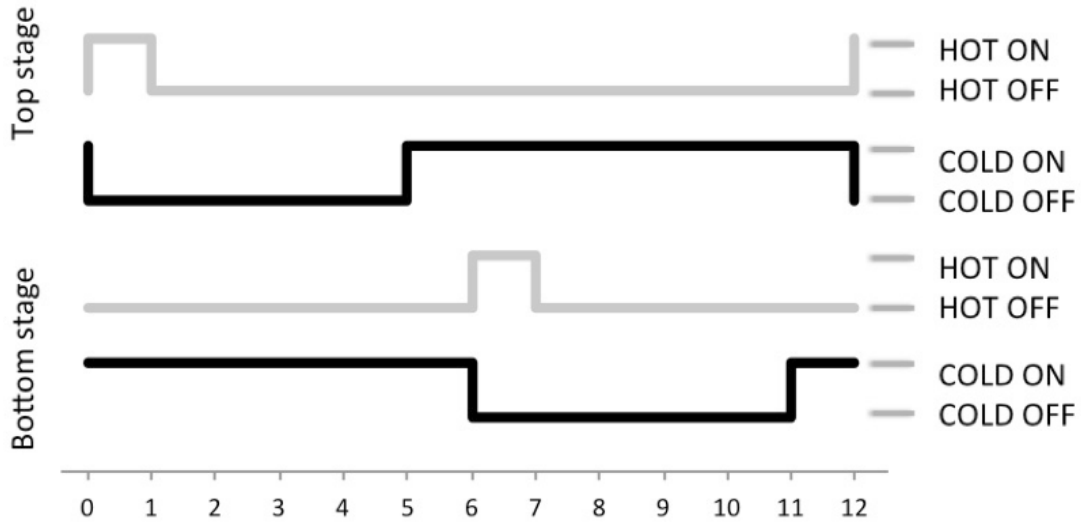

Figure 3.3. Modified timing parameters of Leco quad jet modulator for 12 s contra-directional modulation.

present investigation by using a long modulation period (2 × 6 s = 12 s) but sacrificed modulation ratio as a result. While this leads to a small compromise of first-dimension separation, there is sufficient separation remaining for the proof-of-principle application using reference compounds. More attention to maintaining adequate modulation ratio may be needed for analysis of real samples. A higher modulation ratio could be achieved by slowing down the first dimension separation. The final operating conditions in the present investigation were determined by carefully routing a small length of each of the second-dimension columns (one at a time) outside the GC oven, thereby creating a cold spot. This meant that the second-dimension column separations could be monitored in isolation without having to deconstruct the column configuration, which would have led to changes in flow, and not provided meaningful results. This strategy was very useful for checking the presence (or in this case, absence) of any wrap-around.

A typical chromatogram is shown in **Figure 3.4** for the analysis of laboratory reference compounds. A list of analytes and their respective retention times in the first- and both second-dimension columns is provided in **Table 3.1**. The cyanopropylphenyl dimethyl polysiloxane stationary phase interacts primarily through dispersive forces, while the polyethylene glycol stationary phase has stronger dipolar and hydrogen-bonding interactions [133]. As a result, the primary and secondary alcohols are most strongly retained; alkyl aromatics, ketones and acetates have intermediate retention, while alkanes and halogenated hydrocarbons are eluted quickly from second-dimension *Column 1*. The trifluoropropylmethyl polysiloxane stationary phase has strong dipolar interactions but weak hydrogen-bonding interactions [133]. As a result, the ketones and acetates are more strongly retained

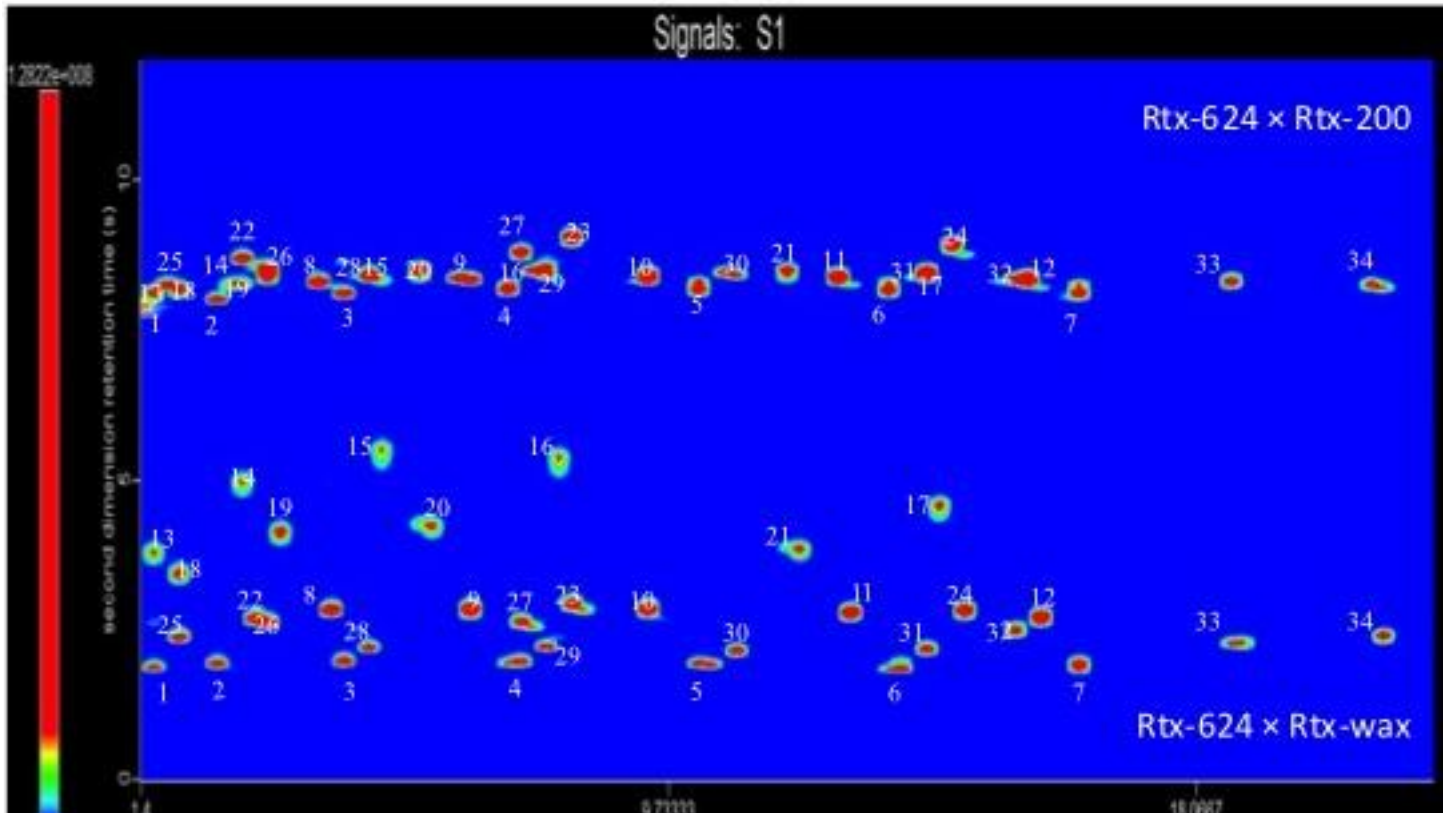

Figure 3.4. The 2-dimensional chromatograms of a 34-component mixture. The separation obtained using cyanopropylphenyl dimethyl polysiloxane × trifluoropropylmethyl polysiloxane appears in the upper portion of the separation space, while the separation achieved using cyanopropylphenyl dimethyl polysiloxane × polyethylene glycol appears in the lower half of the separation space. Refer to Table 4. 1 for compound identification.

Table 3.1. Compiled retention times for a collection of 34 laboratory reference compounds

Compound		$^{1}t_{R}$ (min) Rtx-624	$^{2}t_{R(1)}$ (s) Rtx-wax	$^{2}t_{R(2)}$ (s) Rtx-200
1	*n*-Hexane	1.6	1.88	1.89
2	*n*-Heptane	2.6	1.95	2.01
3	*n*-Octane	4.6	1.99	2.14
4	*n*-Nonane	7.4	2	2.19
5	*n*-Decane	10.2	1.96	2.21
6	*n*-Undecane	13.4	1.86	2.19
7	*n*-Dodecane	16.2	1.92	2.17
8	Toluene	4.4	2.86	2.32
9	Ethylbenzene	6.6	2.89	2.35
10	*n*-propylbenzene	9.4	2.89	2.38
11	Butylbenzene	12.6	2.83	2.41
12	Pentylbenzene	15.6	2.77	2.37
13	Propanol	1.6	3.82	2.13
14	Butanol	3	5.01	2.28
15	Pentanol	5.2	5.52	2.41
16	Hexanol	8	5.43	2.56
17	Octanol	14	4.59	2.45
18	2-Butanol	2	3.47	2.25
19	2-Pentanol	3.6	4.18	2.47
20	2-Hexanol	6	4.27	2.53

21	2-Octanol	11.8	3.89	2.53
22	2-Pentanone	3.2	2.72	2.71
23	2-Heptanone	8.2	2.98	3.08
24	2-Nonanone	14.4	2.86	2.98
25	Ethyl acetate	2	2.39	2.25
26	Propyl acetate	3.2	2.72	2.47
27	Pentyl acetate	7.4	2.69	2.81
28	1-Fluoroheptane	5	2.22	2.41
29	1-Fluorooctane	7.8	2.24	2.56
30	1-Fluorononane	10.8	2.18	2.48
31	1-Fluorodecane	13.8	2.19	2.45
32	1-Chlorodecane	15.2	2.5	2.37
33	1-Bromooctane	18.8	2.32	2.35
34	1-Bromodecane	21	2.44	2.31

than the alcohols. The halogenated hydrocarbons, alkyl aromatics, alcohols and alkanes are poorly retained.

An apex plot for the two GC × GC chromatograms (**Figure 3.5**) further demonstrates the benefit of two dissimilar second-dimension columns, by clearly showing the two-dimensional retention behaviour of the different compound classes present in the sample. The range of secondary retention is much greater on the polyethylene glycol column than the trifluorpropylmethyl polysiloxane. Greater utilisation of the second-dimension separation space should be achieved by use of a thicker film in the trifluorpropylmethyl polysiloxane column. The stationary phase combination in the present study was selected based on Seeley and co-workers GC × 2GC experiments [98,100]. It is possible that other column configurations can be developed to extend the scope of GC × 2GC analysis. Comparison of peak areas of the 34-component mixture for the two second-dimension chromatograms is made in **Figure 3.6**. Absolute peak areas in second-dimension *Column 2* were consistently higher than those in second-dimension *Column 1*. This can be ascribed to slight differences in column dimensions (internal diameter, and/or length) that would lead to an uneven split at the end of the first-dimension column. To overcome this problem, normalised data are presented – where *n*-nonane was treated as an internal standard, and all peak areas are presented as relative peak area (compared to *n*-nonane). In this chart, the consistency of peak areas is greatly improved, with the exception of peak pairs 15/28 (pentanol/fluoroheptane), 16/29 (hexanol/fluorooctane), and 17/31 (octanol/fluorodecane), which were co-eluted from the second-dimension *Column 2*, giving rise to inaccurate peak area measurement using the cyanopropylphenyl × trifluoropropylmethyl polysiloxane column combination. This pleasing result proves the utility of the multiplexed GC × 2GC approach. Multiplexed GC × 2GC approach

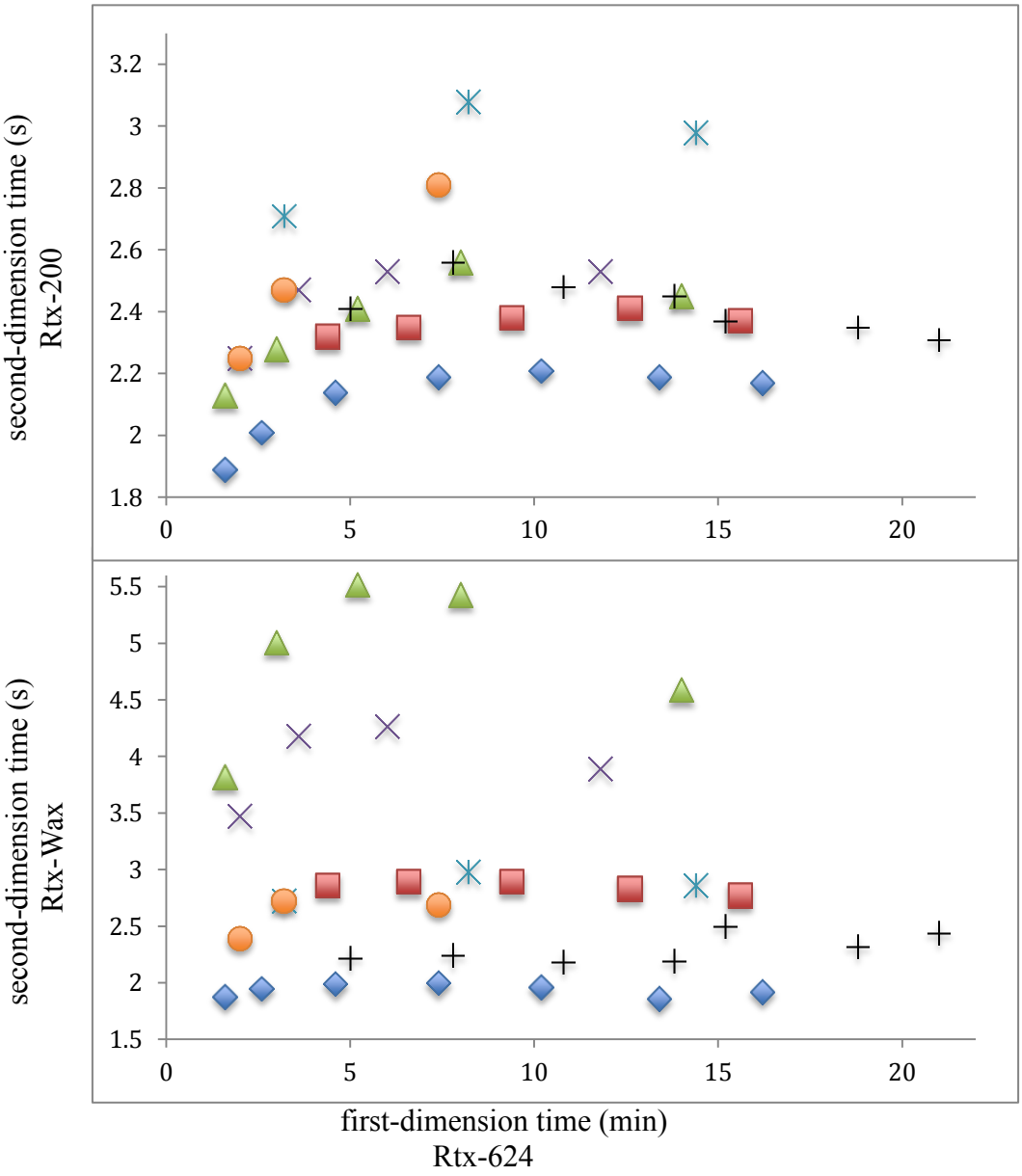

Figure 3.5. Compound classes of the peaks shown in Figure 4. 4. The data are organised according to functional group: ◆ = alkanes, + = halogenated hydrocarbons, ■ = alkyl aromatics, * = acetates, ▲ = 1-alcohols and × = 2-alcohol, ● = 2-ketones

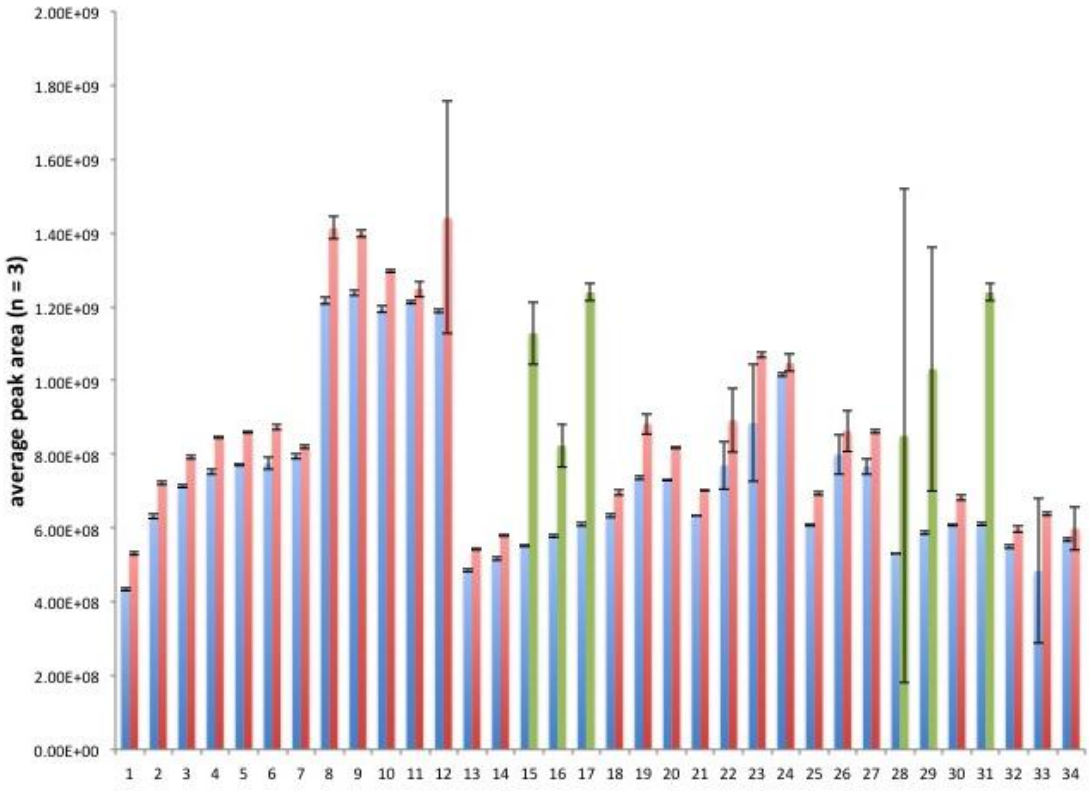

Figure 3.6. Comparison of average relative average peak areas (*n* = 3; compared to (4) *n*-nonane) for the 34 components listed in Table 4. 1. Series 1 is for *Column 1;* Series 2 is for *Column 2*. Co-elution of peaks 15/28 (pentanol/fluoroheptane), 16/29 (hexanol/fluorooctane), and 17/31 (octanol/fluorodecane), which were co-eluted from the second-dimension *Column 2* are indicated by using a different colour. Error bars show 95% confidence interval.

is highly promising for qualitative analysis, since it provides three sets of independent retention times (or retention indices) in a single analysis. Relative peak areas are useful to crosscheck the resolved peaks in each GC × 2GC chromatogram produced during the analysis. Having three sets of retention data should reduce likelihood of false positive identifications. This is evident in Figure 3.6, where co-elutions are exposed that might go unnoticed in a typical GC × GC analysis, where a single second-dimension column is employed. The consistency of relative peak areas for resolved peaks, regardless of second-dimension column, is also encouraging for future use of multiplexed GC × 2GC for quantitative analysis, again due to reduced likelihood of co-elution in two second-dimension columns. The results presented here indicate that it is acceptable to choose relative peak areas from either separation channel to quantify target analytes. Future investigations to couple multiplexed GC × 2GC using contra-directional second-dimension columns with mass spectrometry will be important to realise the full potential and ultimate goal of the work described here. Combination of three retention times (or retention indices) with mass spectrometry should provide near unequivocal assignment of individual peak identity in complex multicomponent samples. The approach developed in the present study showed that this proof-of-principle investigation provides significant impetus for development of GC × 2GC-MS methodology.

3. 4. Conclusion

A multiplexed dual-secondary column GC × 2GC system was developed that splits the first-dimension column effluent into two second-dimension columns with different stationary phases, and recombines the two streams into one detector post-separation. The unique feature of this new approach is that it requires only a single detector. The chromatogram provides complementary information due to the unique

selectivity of the two second-dimension columns. In addition, the approach can be used to generate three unique sets of retention data for all separated analytes. The additional information is especially useful in separating and identifying complex mixtures containing different functional groups. The contra-directional second-dimension column approach needs to be further explored by coupling GC × 2GC with mass spectrometry to take full advantage of its single-detector compatibility. That is, the focus can be placed on serial sample delivery within the MS time frame. Wrap around would be detrimental to multiplexed GC × 2GC methodology, so particular attention need be paid while developing and implementing any new operating conditions.

Chapter 4

Multiplexed dual first-dimension comprehensive two-dimensional gas chromatography – mass spectrometry with contra-directional thermal modulation

This chapter is published:

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4. 1. Introduction

Comprehensive two-dimensional gas chromatography coupled with mass spectrometry (GC × GC-MS) is a highly desirable technology for essential oil characterisation [85,86,89-91,134]. Since many essential oil constituents have similar mass spectral features [35] it is normal practice to obtain retention indices of compounds from one or more different stationary phases to facilitate correct identification of separated compounds [22]. To date many essential oil analysts have utilised dual channel GC [9] to obtain multiple linear retention indices (LRI) on different stationary phases in a single run [5]. The benefit of multiple LRI was very well-illustrated by Bicchi *et al*. who showed that the percentage of correct identifications obtainable through retention indices alone is approximately 65% with one stationary phase, 80% with two different-stationary phases and more than 90% if three different stationary phase columns are employed [22].

GC × GC-MS uses different stationary phases in the first- and second-dimension, however, use of the second-dimension retention time is not well established for compound identification. Although some studies have proposed different methods for calculating retention indices in the second-dimension [135-138], they have not been widely adopted due to their complexity. GC × GC-MS analysis typically depends on the first-dimension retention time and mass spectral data for compound identification [85,86,89-91,134]. Easy access to a second reliable LRI for separated compounds would be highly beneficial for GC × GC-MS separations. Drawing inspiration from previous GC × 2GC investigations [98-100], which use two different second-dimension separation columns and two detectors, we recently introduced a multiplexed, dual second-dimension column GC × 2GC approach that recombines effluent from the two second-dimension columns before analyte detection,

eliminating the need for two detectors. The present investigation builds upon the foundation laid in our earlier work using the principle of contra-directional modulation [139]. A novel approach for multiplexed dual first-dimension GC × GC (2GC × GC), providing dual LRI, coupled with MS is introduced.

Qualitative analysis of *Melaleuca alternifolia* essential oil (Australian tea tree oil) is used to demonstrate the utility of the system developed herein. 2GC × GC-MS analysis with MS database matching and multiple LRI searching provides robust peak assignment based on three orthogonal parameters: MS, LRI_1, and LRI_2. Retention index based searches are performed on LRI data obtained from either of the first-dimension columns, or both of the first-dimension columns by cross searching. Additional supporting information upon which peak identity can be made is based on peak coordinates within the structured chromatograms obtained from the two GC × GC separations.

4. 2. Materials and methods

Chemicals and reagents

Australian tea tree (*M. alternifolia*) essential oil was obtained from a local supermarket (Sandy Bay, Tasmania, Australia) and stored at room temperature for an extended period (beyond the manufacturer's recommended use-by date; SEPT/2011). A C_7-C_{30} *n*-paraffin hydrocarbon mixture (1000 µg/mL; Sigma-Aldrich, Castle Hill, Australia) was used for determination of first dimension LRI. Both the Australian tea tree essential oil and alkane mixture were diluted (1:10, v/v) in dichloromethane (Sigma-Aldrich) prior to GC analyses.

Instrumentation and experimental conditions

All analyses were performed using a Leco GC × GC-FID instrument with an LN2 Cooled Thermal Modulator (Leco Australia, Castle Hill, Australia). The chromatograph was equipped with split/splitless injector, operated with a 20:1 split ratio and inlet temperature of 230 ^{o}C. Injected sample volume was 1 μL. Hydrogen carrier gas was generated using a Parker Balston H2PEM-260 hydrogen generator. For convenience of contra-directional column installation and operation, the auxiliary (second-dimension) column oven was removed from the GC × GC system. All columns were heated using the main GC oven in all analysis. The modulator offset temperature was +15 ^{o}C. Effluent from both secondary columns was monitored by a single FID operated at 100 Hz and 250 ^{o}C. All GC × GC-FID results were collected and processed using Leco ChromaTOF software.

GC × 2GC-FID

GC × 2GC analyses were performed by using the general instrument configuration described in reference [139]. The first dimension column ^{1}D was a 30 m × 250 μm i.d. fused silica capillary coated with 0.25 μm dimethyl polysiloxane (Rtx-1; Restek, Bellefonte, PA, USA). The two second-dimension columns were: $^{2}D_{1}$ 0.4 m × 100 μm i.d. fused silica capillary coated with 0.1 μm polyethylene glycol (Stabilwax; Restek); $^{2}D_{2}$ 0.4 m × 100 μm i.d. fused silica capillary coated with 0.1 μm 50% phenyl, 50% dimethyl polysiloxane (Rxi-17SilMS; Restek). Total carrier gas flow rate was 2.0 mLmin^{-1} (Hydrogen; constant flow). Each of the second-dimension columns operates at Efficiency-Optimised Flow [120] using this flow regime, while the first-dimension column is between Efficiency- and Speed-Optimised Flow. Total modulation period was 6.0 s to maintain appropriate modulation ratio. Custom modulation parameters are provided in **Table 4.1**. The oven temperature program was 40 ^{o}C (0.2 min hold) ramped at 6 ^{o}Cmin^{-1} to 180 ^{o}C. The first-dimension column was connected to the two

second-dimension columns using a 3-port SilFlow connector (SGE Analytical Science, Ringwood, Australia). Flow from the second-dimension columns was recombined using a 3-port SilFlow connector into a 0.2 m × 220 μm i.d. deactivated uncoated fused silica transfer line that terminated at the FID. The second dimension columns were installed contra-directionally in the GC × GC modulator.

2GC × GC-FID

Multiplexed 2GC × GC is achieved by using contra-directional modulation, where two parallel first-dimension columns are installed contra-directionally in the GC × GC modulator as shown in **Figure 4.1**. 2GC × GC analyses were performed by splitting the flow from the inlet into two first-dimension columns by means of a twin-hole graphite ferrule (SGE Analytical Science), all other column connections were made using press tight connectors (Restek). The first dimension columns were installed contra-directionally in the GC × GC modulator. Two first dimension columns were employed in this study; a 30 m × 250 μm i.d. fused silica capillary coated with 0.25 μm dimethyl polysiloxane (DB-1; Agilent Technologies, Mulgrave, Australia); and a 30 m × 250 μm i.d. fused silica capillary coated with 0.25 μm polyethylene glycol (DB-WAX; Agilent Technologies). The second-dimension column was a 0.45 m × 150 μm i.d. fused silica capillary coated with 50% phenyl, 50% dimethyl polysiloxane (Rxi-17SilMS; Restek). Total carrier gas flow rate was 1.5 $mLmin^{-1}$ (Hydrogen; constant flow), which provides Speed-Optimised Flow in the second column. Total modulation period was 3.0 s to maintain appropriate modulation ratio, and the oven temperature program was 40 ^{o}C (0.2 min hold) ramped at 7 $^{o}Cmin^{-1}$ to 220 ^{o}C (3 min hold). Custom modulation parameters are provided in **Table 4.1**.

GC × GC-FID

GC × GC analyses were performed using the columns as 2GC × GC experiments, except they were installed in the regular configuration through the GC × GC modulator and all column connections were made using press-tight connectors (Restek). The flow rate was 1.0 $mLmin^{-1}$ for all experiments. A modulation period of 3.0 s (with default modulation parameters) was employed and the temperature program was 40 ^{o}C (0.2 min hold) ramped at 5.8 $^{o}Cmin^{-1}$ to 220 ^{o}C (3 min hold).

2GC × GC-MS

2GC × GC-MS analysis was performed using the Leco GC × GC system described above, which was connected *via* an externally controlled heated transfer line, to the heated MS transfer line of an Agilent 5975C VL MSD (Agilent Technologies). The external heated transfer line was a 155 mm Agilent LTM transfer line, which was controlled with an LTM Controller (Agilent Technologies). The external heated transfer line and heated MS transfer line temperatures were 200 ^{o}C. The MS detector was operated with a source temperature of 150 ^{o}C, quadrupole temperature 230 ^{o}C**,** with a detector voltage of 70 eV. ChemStation software (Agilent Technologies) was used to acquire MS data in the fast scanning mode with a reduced mass scan range of 40-220 *m/z,* yielding a detector sampling rate of 25.95 Hz. Data acquisition was triggered by a start-out signal from a remote port provided by the Leco GC × GC modulator. GC columns and other operating conditions were identical to those described above for the 2GC × GC-FID experiments. MS data were visualised using Transform (Fortner Research, Boulder, CO, USA). Mass spectra were matched to the Terpene Library (containing mass spectra of essential oil components compiled by Robert P. Adams, Baylor University Plant Biotechnology Center). Further analysis of 2GC × GC-MS results using interactive LRI filters was performed using Aroma Office 2D Software (Gerstel K.K., Tokyo, Japan). This software is a searchable

database with more than 100,000 entries, containing LRI information for a wide range of aroma compounds from many literature references[140,141].

4. 3. Results and discussion

4. 3. 1 Implementation and benefits of 2GC × GC system

In the present investigation, two long primary columns are installed as parallel first-dimension columns. This column configuration mirrors a previous GC × 2GC study that used two short second-dimension columns. While column installation is straightforward, a key practical consideration for contra-directional modulation approach relates to column alignment within the modulator. Proper column alignment is critical to ensure that neither of the two column segments is allowed to block the jet of cool gas onto the other column. **Figure 4.2** compares the multiplexed comprehensive two-dimensional chromatograms obtained from the analysis of *M. alternifolia* essential oil using GC × 2GC and 2GC × GC. There are advantages and disadvantages of each of these multiplexed approaches. The first advantage of GC × 2GC is that peaks in both chromatograms have matching first-dimension retention times. This makes the distribution of peaks in GC × 2GC more intuitive than the distribution observed in 2GC × GC, but this is also disadvantageous because GC × 2GC only provides a single first-dimension LRI. Having two dissimilar second-dimension columns also creates optimisation challenges to avoid wrap-around. A single second-dimension column greatly alleviates this challenge. Next, it is important to use a non-polar stationary phase column in the first-dimension for GC × 2GC experiments, since retention indices are more rugged on non-polar stationary phases. However the requirement to use a non-polar stationary phase first-dimension column means that second-dimension columns can only be more polar than the first-

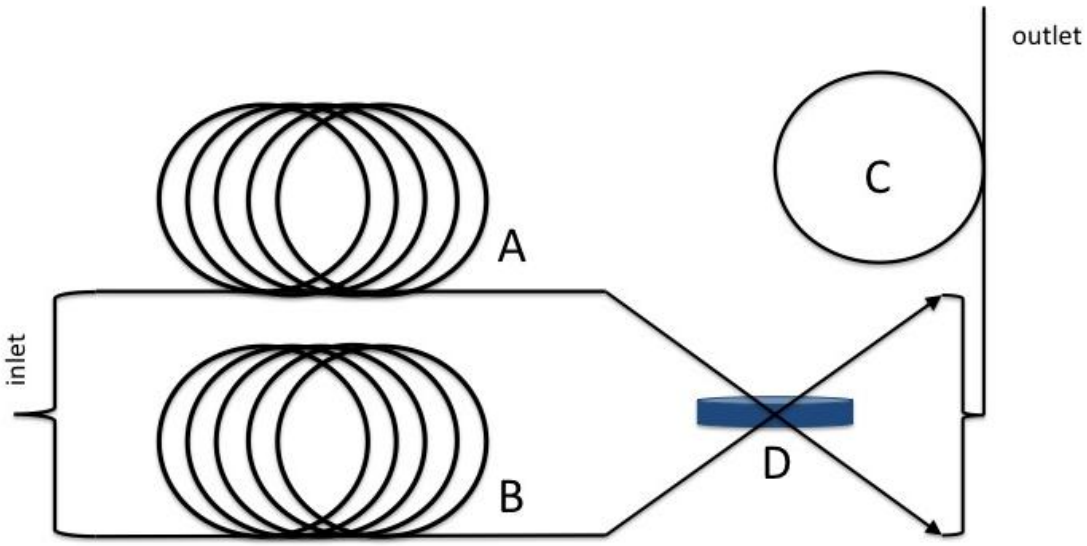

Figure 4.1. Illustration of the column configuration used for multiplexed 2GC × GC.

A, first-dimension *Column 1*: polyethylene glycol; B, first-dimension *Column 2:* dimethyl polysiloxane; C, second-dimension column: 50% phenyl, 50% dimethyl polysiloxane; D, dual stage thermal modulator.

Table 4.1. Advanced modulation parameters for contra-directional modulation.

GC×2GC				
FIRST STAGE	Cool Time Till Off	Cool Off Time	Heat Time Till On	Heat On Time
	3000 ms	2500 ms	2500 ms	3500 ms
SECOND STAGE	Cool Time Till On	Cool On Time	Heat Time Till Off	Heat Off Time
	3000 ms	1000 ms	1000 ms	5000 ms
2GC×GC				
FIRST STAGE	Cool Time Till Off	Cool Off Time	Heat Time Till On	Heat On Time
	1500 ms	1200 ms	1200 ms	1800 ms
SECOND STAGE	Cool Time Till On	Cool On Time	Heat Time Till Off	Heat Off Time
	1500 ms	1200 ms	1200 ms	1800 ms

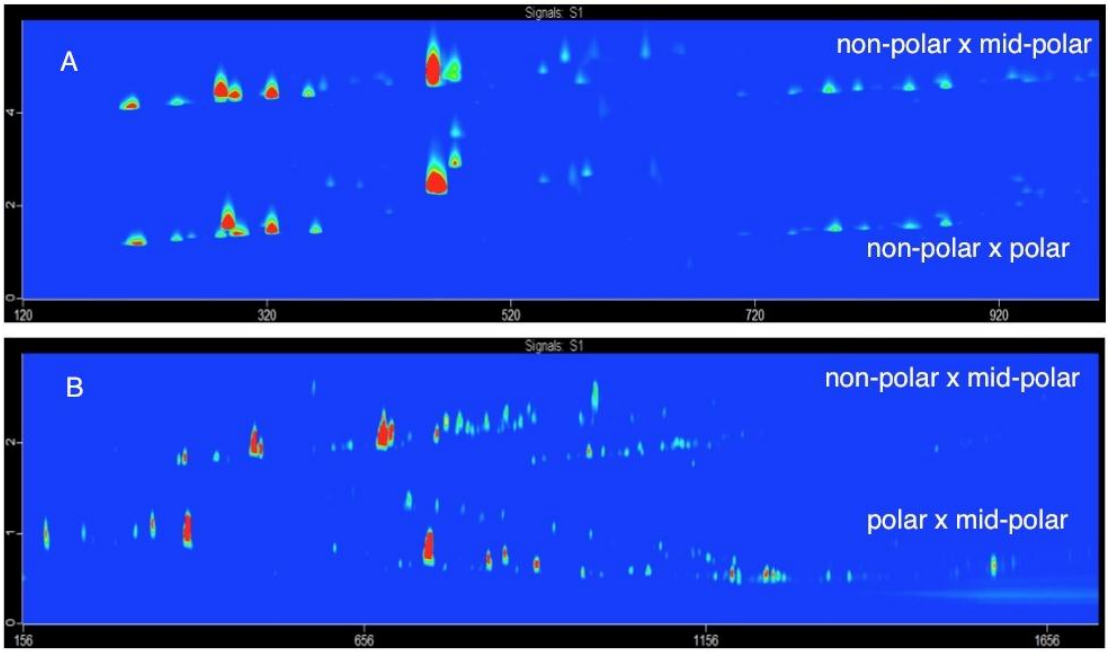

Figure 4.2. Multiplexed two-dimensional separation space for the separation of *M.alternifolia* essential oil. (A) Separations obtained using GC × 2GC, (B) separations were obtained using 2GC × GC.

dimension column. As the purpose of the dual second-dimension separations is to resolve any co-eluted compounds, this restriction limits peak spreading throughout the two-dimensional separation space. Indeed the individual GC × GC chromatograms produced by the GC × 2GC approach, shown in Figure 4.2A exhibit more similarities than differences. A column ensemble comprising one long first-dimension column and two short second-dimension columns in GC × 2GC is not optimal for fully harnessing the complementary selectivity of the three stationary phases. Conversely, using two long first-dimension columns and one short second-dimension column provides a demonstrable improvement over GC × 2GC in utilising the available separation space. Retention of any given solute in the second-dimension of temperature-programmed GC × GC analysis is dependent upon the solute's interaction with the first- and second-dimension stationary phases [30]. Having two first-dimension columns in the 2GC × GC experiment is valuable since they make different contributions to retention in the second-dimension column. In the top half of Figure 4.2B (*dimethyl polysiloxane* × *50% phenyl, 50% dimethyl polysiloxane* column combination; hereafter referred to as *non-polar* × *mid-polar*) analytes are strongly retained in the second-dimension column due to strong solute-stationary phase interactions with the second-dimension column stationary phase. The separation in the bottom half of Figure 4.2B, which represents a column combination of *polyethylene glycol* × *50% phenyl, 50% dimethyl polysiloxane* (hereafter referred to *polar* × *mid-polar*), demonstrates how a stronger interaction with the first-dimension column stationary phase decreases retention in the second-dimension. The interaction of δ-elemene (peak 32) and cyclosativene (peak 37) illustrates this effect in the two chromatograms shown in Figure 4.2B. Although GC × 2GC experiments previously described in the literature demonstrate beneficial class discrimination for homologous

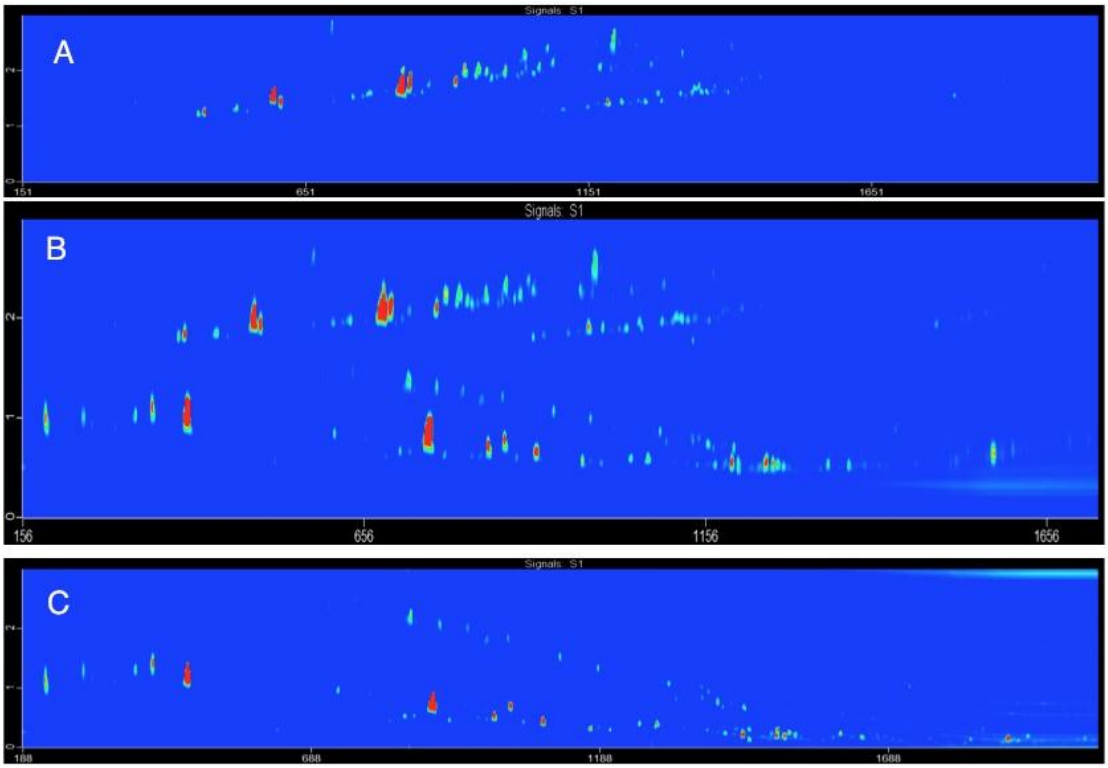

Figure 4.3. Two-dimensional separation space for GC × GC and 2GC × GC analyses of *M. alternifolia* essential oil. (A) separation obtained using GC × GC (non-polar × mid-polar), (B) separations obtained using 2GC × GC, (C) separation obtained using GC × GC (polar × mid-polar).

series [98-100,139], 2GC × GC is more appropriate for analysis of genuinely heterogeneous multicomponent samples. One may postulate that greater utilisation of the two-dimensional separation space (compared to Figure 4.2A) would be achieved by using a *mid-polar* × *non-polar* plus *mid-polar* × *high-polar* GC × 2GC combination, but the benefit of recording reliable LRIs would be diminished.

Figure 4.3 compares the chromatograms obtained from 2GC × GC and conventional GC × GC analyses of *M. alternifolia* essential oil. The two GC × GC analyses were performed using the same columns as the 2GC × GC experiment. The columns were not multiplexed in these experiments; they were installed in a conventional GC × GC configuration. It is evident that GC × GC chromatogram integrity is preserved in the 2GC × GC experiment. The corresponding chromatograms exhibit striking resemblance and the number of peaks separated in each GC × GC analysis is similar to the corresponding portion of the 2GC × GC analysis. Importantly, the 2GC × GC experiment maintains an appropriate number of modulation slices in line with accepted guidelines. This ensures sufficient peak sampling to minimise modulation-induced loss of first-dimension resolution [71]. By employing a total modulation period of 3 s the modulation ratio is at least 2 in the 2GC × GC experiments, which is suitable for semi-quantitative screening purposes according to the recommendations of Khummueng *et al*. [73]. By nature of the contra-directional modulation approach, the second-dimension separation needs to be twice as fast the normal requirement for GC × GC. A fast second-dimension separation is especially important in 2GC × GC to avoid wrap-around, which would cause the two separation windows in the 2GC × GC chromatogram to overlap. Wrap-around is quite easily avoided by using short second-dimension columns and applying speed-optimised Flow (for the second-dimension column). Fine-tuning the separation space can be achieved by

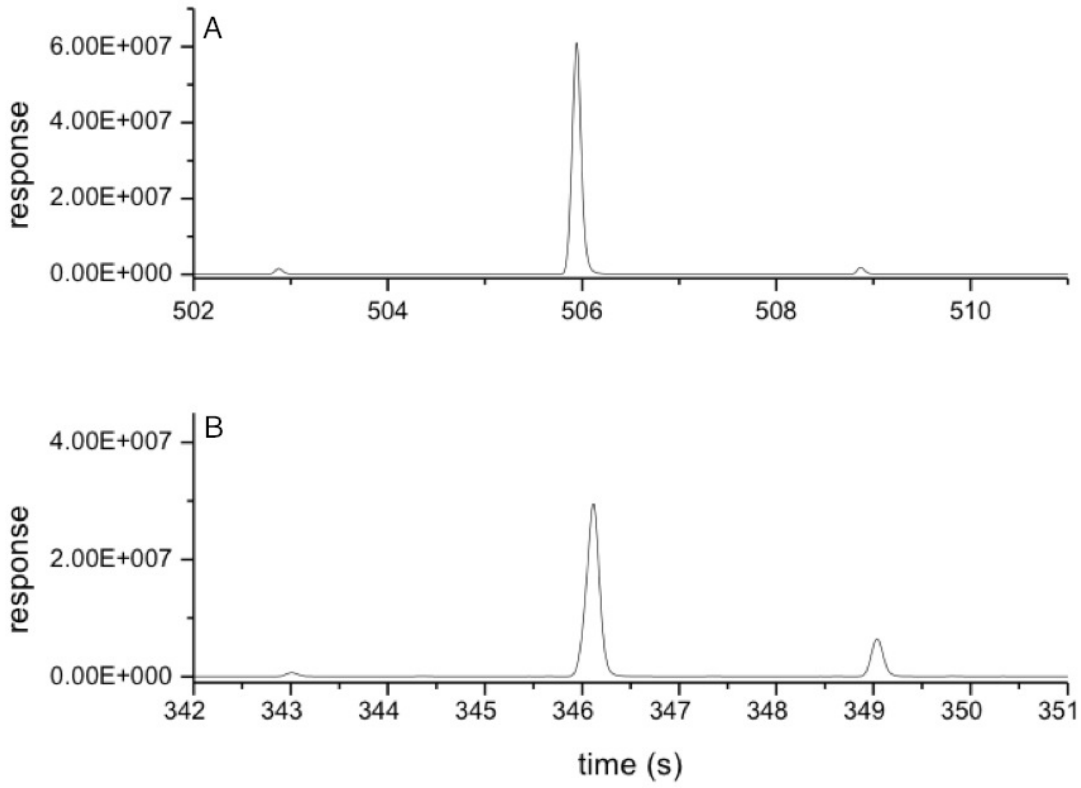

Figure 4.4. 3 s modulated peak pulses in phase obtained from 2GC × GC for 1,8-cineole (peak 7). (A) non-polar × mid-polar, (B) polar × mid-polar.

manipulating the initial temperature and temperature ramp(s). A 1.5 s separation window is sufficient for the sample considered in the present investigation. Unconverted GC × GC chromatograms of 1,8-cineole (peak 7) obtained from the 2GC × GC experiment are shown in **Figure 4.4**. The figure shows that each of the multiplexed GC × GC separations produces three modulated pulses above baseline for 1,8-cineole. Measured peak areas are 718 ± 8 and 581 ± 7 (n = 4) for 1,8-cineole peaks in the *non-polar* × *mid-polar* and *polar* × *mid-polar* 2GC×GC separations respectively. Although these peak area values are statistically significantly different ($p < 10^{-6}$) due to a slightly uneven split between the two first-dimension columns, no further effort was made to equalise the split ratio in the present study, since the aim was to demonstrate qualitative performance benefits. Previous work discussed use of normalised peak areas for semi-quantitative analysis in GC × 2GC experiments [139] and this is also likely to be beneficial for the current configuration.

4. 3. 2 Coupling of 2GC × GC system with mass spectrometry (2GC × GC-MS)

Although time of flight mass spectrometry (TOF-MS) is the mass selective detector of choice for GC × GC-MS, quadrupole MS instruments have been successfully used for this purpose [89,90,142,143], usually by reducing the scan mass range to obtain a reasonable data acquisition rate [89]. The present investigation applied a reduced mass scan range from 40-220 *m/z* with fast scanning mode providing a data acquisition rate of *ca*. 26 Hz. This data acquisition rate is adequate for proof-of-principle qualitative analysis, although a faster instrument would be required to realise the full potential of 2GC × GC-MS analysis for quali-quantitative analysis (identification and amount). A 2GC × GC-MS peak apex plot of *M. alternifolia* essential oil along with peak numbers in both GC × GC separations is provided in

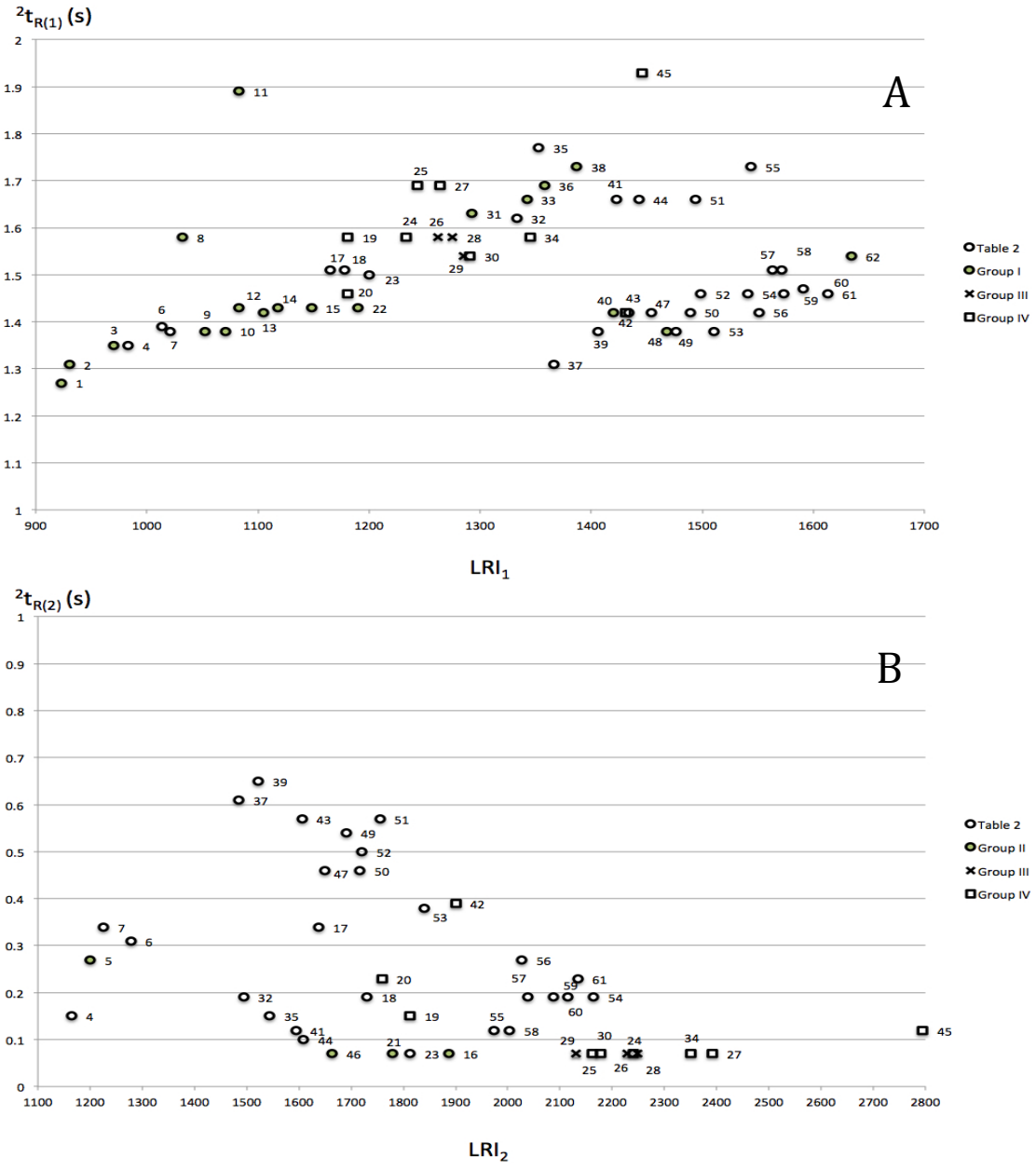

Figure 4.5. Peak apex plots showing coordinates of peak maxima from 2GC × GC-MS analysis of *M. alternifolia* essential oil. (A) non-polar × mid-polar, (B) polar × mid-polar. Peak numbers correspond to those in Tables 4.2 and 4.3.

Figure 4.5. The 2GC × GC-MS chromatogram is provided in the appendix (see Figure A1).

Primary identification of separated compounds was carried out by MS_1 library searching peaks in one of the multiplexed GC × GC separations, using a minimum similarity index threshold of ≥ 80 %. Secondary confirmation of MS_1 based peak identification was achieved by comparing the first-dimension LRI_1 of each peak with literature LRI using AROMA Office 2D software. The threshold for permitted LRI_1 shift was ± 10 for the dimethyl polysiloxane stationary phase column. The next level of confirmation was achieved by using LRI_2 to locate a peak in the alternate multiplexed GC × GC separation. To this end, any peak name that remained after MS_1 library search and LRI_1 filter was input (name-search) in Aroma Office 2D. Name-search responses were limited to the polyethylene glycol stationary phase. Here, a full list of LRI_2 from the database is reported for entries matching that compound name. The mean LRI_2 reported by Aroma Office 2D is used to perform a reverse-LRI_2 search on the alternate first-dimension column, using ΔLRI_2 of ± 50. This reverse LRI_2 search narrows the search area in the two-dimensional separation space for MS_2 library searching. Additional information, such as relative peak intensity and second-dimension relative retention within the structured two-dimensional separation space is used to assist peak selection for MS_2 library searching. Final confirmation is performed by MS_2 library searching using the same similarity threshold as the MS_1 library search. A peak name is only added to the list of identified components if the thresholds for MS_1 search, LRI_1 filter, LRI_2 search, and MS_2 search are satisfied. **Table 4.2** provides details of 27 components identified in *M. alternifolia* essential oil, using the four peak assignment criteria. The identified components are also shown in a peak-apex plot (Figure 4.5**)** to permit cross-referencing with the 2GC × GC

chromatogram (Figure 4.3).

Table 4.3 provides a list of 35 *M. alternifolia* components that failed at least one identification criterion above. Despite failing one or more criteria, many of these components were identified using alternative strategies. These components are grouped according to the strategy used for peak assignment. Group I comprises 19 components whose identity was indicated by MS_1 library searching and confirmed by LRI_1. Similarly, Group II contains 4 components that were identified by MS_2 library searching and confirmed by LRI_2. The components assigned to Groups I and II were not found in the alternate GC × GC separation within the corresponding match thresholds. Broadening the ΔLRI threshold did not lead to further confirmatory results. Limited further confirmation of peak identity of these 35 components is largely due to peak co-elution, which leads to poor MS library search results. For instance, limonene (peak 5) was positively identified using the polyethylene glycol stationary phase first-dimension separation using MS_2 and LRI_2. The Aroma Office 2D software indicates a LRI_1 of 1014 for the dimethyl polysiloxane column. Thus this peak must be eluted between *p*-cymene (peak 6 LRI_1 1014) and 1,8-cineole (peak 7 LRI_1 1021) in the chromatogram, but it is obscured in the separation. This problematic peak triplet has been previously reported in the same essential oil using one-dimensional GC-MS analysis [23].

Like Group I components, identity of the three components assigned to Group III was indicated by MS_1 library searching and confirmed by LRI_1. None of these components were located within the allowable ΔLRI_2 windows in the polyethylene glycol stationary phase first-dimension separation space. However, in this case the widely discussed class-separation leads to further corroboration of peak assignment.

Table 4.2. Peak assignments of 27 components for which all search criteria were satisfied. Numbers in the Table correspond to those in Figure 4.5. MS library match quality refers to comparison with the Terpene Library.

Peak No	Peak Assignment	Column Set 1					Column Set 2				
		% match MS_1	LRI_{1obs}	$^2t_{R(1)}$	LRI_{1lit}	$\Delta(LRI_{obs}-LRI_{lit})_{(1)}$	% match MS_2	LRI_{2obs}	$^2t_{R(2)}$	LRI_{2lit}	$\Delta(LRI_{obs}-LRI_{lit})_{(2)}$
4	myrcene	95	983	1.35	983	0	93	1164	0.15	1160	4
6	*p*-cymene	96	1014	1.39	1014	0	96	1278	0.31	1271	7
7	1,8-cineole	94	1021	1.38	1021	0	95	1225	0.34	1212	13
17	terpinen-4-ol	82	1165	1.51	1164	1	88	1638	0.34	1628	10
18	α-terpineol	93	1178	1.51	1177	1	95	1731	0.19	1729	2
23	*cis*-carveol	82	1200	1.5	1202	-2	81	1813	0.07	1834	-21
32	δ-elemene	86	1333	1.62	1338	-5	91	1494	0.19	1473	21
35	α-copaene	85	1353	1.77	1357	-4	87	1544	0.15	1523	21
37	cyclosativene	93	1366	1.31	1372	-6	89	1485	0.61	1481	4
39	α-gurjunene	93	1406	1.38	1411	-5	90	1522	0.65	1531	-9
41	*trans*-α-	84	1423	1.66	1428	-5	86	1594	0.12	1576	18

	bergamotene										
43	β-caryophyllene	95	1434	1.42	1438	-4	93	1606	0.57	1601	5
44	aromadendrene	88	1443	1.66	1448	-5	87	1609	0.1	1605	4
47	allo-aromadendrene	92	1454	1.42	1458	-4	91	1650	0.46	1650	0
49	γ-muurolene	84	1476	1.38	1480	-4	85	1691	0.54	1691	0
50	valencene	89	1489	1.42	1493	-4	88	1717	0.46	1726	-9
51	bicyclogermacrene	82	1494	1.66	1498	-4	84	1755	0.57	1740	15
52	α-muurolene	82	1499	1.46	1504	-5	89	1721	0.5	1725	-4
53	*trans*-calamenene	95	1511	1.38	1518	-7	90	1840	0.38	1844	-4
54	spathulenol	84	1541	1.46	1547	-6	86	2165	0.19	2147	18
55	*trans*-nerolidol	82	1544	1.73	1549	-5	82	1974	0.12	1959	15
56	caryophyllene oxide	88	1551	1.42	1557	-6	89	2027	0.27	2009	18
57	globulol	93	1563	1.51	1568	-5	88	2038	0.19	2060	-22
58	cedrol	90	1572	1.51	1577	-5	84	2004	0.12	2046	-42
59	guaiol	86	1573	1.46	1575	-2	87	2115	0.19	2096	19

60	elemol	90	1591	1.47	1595	-4	84	2088	0.19	2083	5
61	τ-cadinol	80	1613	1.46	1617	-4	82	2134	0.23	2167	-33

Table 4.3. Peak assignments of 35 components for which at least one search criterion was not satisfied. Numbers in the Table correspond to those in Figure 4.5. MS library match quality refers to comparison with the Terpene Library.

Peak No	Peak Assignment	Column Set 1					Column Set 2				
		% match MS_1	LRI_{1obs}	$^2t_{R(1)}$	LRI_{1lit}	$\Delta(LRI_{obs}-LRI_{lit})_{(1)}$	% match MS_2	LRI_{2obs}	$^2t_{R(2)}$	LRI_{2lit}	$\Delta(LRI_{obs}-LRI_{lit})_{(2)}$
Group I											
1	α-thujene	96	923	1.27	923	0	-	-	-	1021	-
2	α-pinene	99	930	1.31	930	0	-	-	-	1022	-
3	sabinene	98	970	1.35	970	0	-	-	-	1119	-
8	*trans*-β-ocimene	83	1032	1.58	1032	0	-	-	-	1250	-
9	γ-terpinene	83	1052	1.38	1051	1	-	-	-	1253	-
10	terpinolene	89	1071	1.38	1070	1	-	-	-	1278	-
11	linalool	91	1083	1.89	1082	1	-	-	-	1546	-
12	*cis*-sabinene hydrate	87	1083	1.43	1082	1	-	-	-	1515	-
13	allo-ocimene	89	1105	1.42	1106	-1	-	-	-	1380	-

14	neo-allo-ocimene	86	1118	1.43	1117	1	-	-	-	1421	-
15	*cis*-sabinol	86	1148	1.43	1147	1	-	-	-	1731	-
22	tenol	86	1190	1.43	1191	-1	-	-	-	1794	-
31	*trans*-pinocarvyl acetate	81	1293	1.63	1296	-3	-	-	-	1764	-
33	α-cubebene	87	1342	1.66	1348	-6	-	-	-	1466	-
36	α-ylangene	89	1358	1.69	1363	-5	-	-	-	1483	-
38	β-cubebene	93	1387	1.73	1392	-5	-	-	-	1533	-
40	β-gurjunene	85	1420	1.42	1424	-4	-	-	-		-
48	virdiflorene	86	1468	1.38	1473	-5	-	-	-	1697	-
62	α-eudesmol	81	1634	1.54	1636	-2	-	-	-	2191	-
Group II											
5	limonene	-	-	-	1014	-	96	1200	0.27	1199	-1
16	*p*-cymen-8-ol	-	-	-	1163	-	93	1887	0.07	1857	-30
21	trans-piperitol	-	-	-	1204	-	82	1779	0.07	1747	-32
46	α-humulene	-	-	-	1449	-	85	1663	0.07	1663	0

Group III											
26	*cis*-verbenyl acetate	84	1262	1.58	1264	2	82	2228	0.07	-	-
28	iso-bornyl acetate	87	1275	1.58	1278	3	84	2248	0.07	1578	670
29	myrtenyl acetate	83	1285	1.54	1290	5	81	2131	0.07	1690	441
Group IV											
19	19	-	1181	1.58	-	-	-	1813	0.15	-	-
20	20	-	1181	1.46	-	-	-	1759	0.23	-	-
24	24	-	1233	1.58	-	-	-	2240	0.07	-	-
25	25	-	1244	1.69	-	-	-	2162	0.07	-	-
27	27	-	1264	1.69	-	-	-	2392	0.07	-	-
30	30	-	1291	1.54	-	-	-	2177	0.07	-	-
34	34	-	1345	1.58	-	-	-	2350	0.07	-	-
42	42	-	1431	1.42	-	-	-	1900	0.39	-	-
45	45	-	1446	1.93	-	-	-	2795	0.12	-	-

Class/structure association is often not readily apparent in essential oil analyses due to the heterogeneity of the samples, but polar first-dimension columns lead to some useful class information [144]. For example, the terpene esters that are shown as crosses in Figure 4.5 were not found within expected ΔLRI_2 in the polyethylene glycol separation space. Utilising the information provided by the structured separation space to indicate the compound class of unknown peaks, and using their relative peak intensities as a further indicator, narrows the candidate list of unassigned peaks for further MS_2 library searching. For example, peak assignment of *cis*-verbenyl acetate (peak 26) was achieved using this strategy. Absolute peak areas of these peaks are 4.3×10^8 and 4.2×10^8 arbitrary units respectively (2GC × GC-FID data). Peak assignment is justified by MS_1 match = 84% and $\Delta LRI_1 = 1262$, and MS_2 library search upon the remaining unassigned candidate peaks giving an MS match = 82% for the peak with $LRI_2 = 2228$ (spectra matching data provided in the appendix A2).

Finally **Group IV** represents a subset of the remaining unassigned components in *M. alternifolia* essential oil. While the identity of these components remains unknown it is still possible to locate the respective peak in the alternate GC × GC separation using a strategy similar to that described for Group III. These unknowns were cross-referenced in the two GC × GC chromatograms by using relative peak intensities as the primary criterion for narrowing the candidate list and matching the respective MS spectra from each of the multiplexed separations (provided in the appendix A5-A13).

4. 4. Conclusion

A multiplexed dual-primary column 2GC × GC-MS system was developed that splits the primary flow into two first-dimension columns and recombines the two streams

into a single second-dimension column, which ultimately ends at a single detector. The unique feature of this approach is that it is able to generate two LRIs and two second-dimension retention ordinates from a single injection, while using a single detector. The analysis of *M.alternifolia* essential oil shows the complementary selectivity of each column with two different first-dimension column LRIs obtained for each analyte. Furthermore, the contra-directional modulation regime in 2GC × GC maintains information content of two individual GC × GC analyses, without the need for dual injection. Forty-nine components were identified using the *non-polar* × *mid-polar* column combination and 34 components were positively identified using the *polar* × *mid-polar* column combination. There were 22 unique compounds identified using the *non-polar* × *mid-polar* column combination and 7 unique compounds identified using the *polar* × *mid-polar* column combination. It is understandable that the former combination provides superior identification since non-polar retention indices are more reliable. Of these identified components, 27 peak assignments were corroborated by positive identification in both of the multiplexed separations. This aspect is the most important benefit of the multiplexed GC × GC-MS approach. This 2GC × GC-MS system with Aroma Office 2D LRI library has proven to be very effective for reliable identification of *M. alternifolia* essential oil constituents.

Chapter 5

Multiplexed dual channel gas chromatography-mass spectrometry (2GC-MS) with contra-directional thermal modulation

5. 1. Introduction

Comprehensive two-dimensional gas chromatography (GC × GC) is a well-known technique, but the essential oil industry relies on the conventional single-dimensional GC-MS for the characterisation of their samples. The interactive use of retention indices from GC with mass spectra data is the usual method of choice for reliable identification of separated compounds. However, the Analytical Methods Committee of the Royal Society of Chemistry and researchers in the field of essential oil analysis recommend the advantages of obtaining these retention indices on two or more different stationary phases to enhance confidence in peak assignments [22,145]. There are two possible reasons for this recommendation. Firstly, many essential oil constituents have similar retention behaviour on certain stationary phases and, secondly, many of them have similar mass spectra that may lead to false assignment of the peaks. There are many articles dealing with the interactive use of retention indices on different stationary phases incorporated with mass spectra data used for essential oil analyses [14,23-25]. Typically single-dimensional GC analyses were run separately to obtain multiple retention indices and MS data. In all cases more accurate qualitative and quantitative analysis about the sample was obtained [14,23-25]. Unfortunately, this method requires multiple analyses, which ultimately increases the time and the cost of analysis.

The introduction of the fused silica capillary column [146] makes it easy to connect two or more columns in the same injection port and led researchers to develop dual-channel GC [9]. In this technique, the sample is introduced through an injection port and the flow is split and directed into two columns with different stationary phases that terminate each with separated detectors. The combination of a polar and non-polar column is used because of their quite different separation characteristics. A dual

channel gas chromatograph using a 100 % dimethyl polysiloxane and polyethylene glycol (both 60 m × 0.25 mm × 0.25 μm) for the analysis of *Achillea millefolium L.* essential oil has been reported [26], in which 56 compounds present in the sample were reliably characterised by the use of two retention indices with mass spectral information. Similar analyses were also reported for the analysis of essential oils from Greek oregano clones [27], *Lavandula angustifolia* and the Lavandin hybrids "*super*" and "*special*" grown in Greece [28]. Unfortunately, all these studies utilised non-specific detectors like FID for the analysis and then incorporate MS data obtained separately for confirmation. The coupling of two MS in such instrumentation is a great challenge for many laboratories. Furthermore, this dual channel system requires two channels of integration and data processing competence. It requires the two channels to be used in a synchronised mode and employ detectors with the same sensitivity; otherwise quantitative analysis may produce erroneous results [9].

The present investigation explores the principle of contra-directional modulation and a novel multiplexed dual channel GC (2GC) approach coupled with mass spectrometry detection. Qualitative analysis of parsley essential oil is used to demonstrate the utility of the system developed herein. Further stimulus to implement 2GC-MS analysis is availability of retention indices of odour active compounds in a single database called Aroma Office 2D [140,141]. A cross-search of multi-retention indices combined with MS database matching provides more accurate peak assignment based on three orthogonal parameters: MS, LRI_1, and LRI_2.

5. 2. Experimental

Samples and sample preparation

Parsley essential oil was provided by Essential Oils of Tasmania (Kingston, Australia). The sample was obtained by steam distillation. An *n*-paraffin hydrocarbon mixture in the range of C_7-C_{30} was obtained from Sigma-Aldrich in purity exceeding 97% where available for determination of linear retention indices (*LRI*). Both the parsley essential oil and alkane mixture were diluted (1:10, v/v) in dichloromethane prior to GC analyses.

Instrumentation

2GC-MS analysis

2GC-MS analyses were performed using a Leco GC × GC instrument with LN2 Cooled Thermal Modulator (Leco Australia, Castle Hill, Australia), which was connected *via* an externally controlled heated transfer line, to the MS transfer line of an Agilent 5975C VL MSD (Agilent Technologies). The external heated transfer line was a 155 mm Agilent LTM transfer line that was controlled with an LTM Controller (Agilent Technologies). The external heated transfer line and MS transfer line temperatures were 200 ^{o}C. The MS detector was operated with a source temperature of 150 ^{o}C, quadrupole temperature 230 ^{o}C**,** with a detector voltage of 70 eV. ChemStation software (Agilent Technologies, Version G1701EA E.01.00.237) was used to acquire MS data in the fast scanning mode with a reduced mass scan range of 40-220 *m/z,* yielding a detector sampling rate of 25.95 Hz. Data acquisition was triggered by a start-out signal from a remote port provided by the Leco GC × GC modulator. The chromatograph was equipped with split/splitless injector, operated in split mode throughout (20:1) and 230 ^{o}C. Sample volume was 1 µL injected. Hydrogen carrier gas was generated using a Parker Balston H2PEM-260 hydrogen generator. The two capillary columns were installed contra-directionally in the GC × GC modulator. Two columns were employed throughout, a 30 m × 250 µm i.d. fused

silica capillary coated with 0.25 μm dimethyl polysiloxane (DB-1; Agilent Technologies, Mulgrave, Australia); and a 30 m × 250 μm i.d. fused silica capillary coated with 0.25 μm polyethylene glycol (DB-WAX; Agilent Technologies). Total carrier gas flow rate was 1.0 mL/min (Hydrogen; constant flow). Total modulation period was 3.0 s and the oven temperature program was 40 ^{o}C (0.2 min hold) to 220 ^{o}C (3 min hold) at 3 ^{o}C/min. For convenience of contra-directional column installation and operation, the auxiliary (second-dimension) column oven was removed from the GC × GC system. All columns were heated using the main GC oven in all analysis. Modulation timing parameters are described in Results and Discussion section. The modulator offset temperature was +15 ^{o}C.

Data analysis

ChemStation software was used for collecting the data and Transform software (Fortner Research, Boulder, CO USA) was used for generating the chromatogram. Mass spectra were matched to the Terpene Library (The Terpene Library contains mass spectra of essential oil components compiled by Robert P. Adams, Baylor University Plant Biotechnology Center). Further analysis of 2GC-MS results, by applying interactive retention index filters was performed using Aroma Office 2D Software (Gerstel K.K., Tokyo, Japan)". This software is a searchable database, which contains LRI information for a wide range of odour compounds from many literature references [140]. The software provides accurate searches through the use of retention indices and a database of over 100,000 entries [141].

5. 3. Results and discussion

Multiplexed dual channel 2GC - MS was performed by employing contra- directional modulation by installing the two columns contra-directionally in the Leco GC × GC dual stage modulator (**Figure 5.1**). A 3 s modulation period is employed herein using the timing parameters provided in **Table 5.1**. The two different columns provide complementary separation of the injected sample, while detection is achieved using a single MS. Unlike previous chapters, there is no second-dimension column employed, so the separations are similar to single-dimensional chromatograms, the only difference is using a dual stage modulator to modulate the peaks eluted from both columns to achieve 2GC-MS. The conditions used in the present investigation are drawn from those often used in essential oil analysis, however, one could choose any column dimensions or experimental conditions for 2GC-MS analysis.

The present study reduced the mass scan range (40 to 220 m/z) and operated in a fast scanning mode providing 26 Hz. Despite only allowing the collection of ~ 4 data points (**shown in Figure 5.2**) for each narrow peak eluted after the modulation, high quality mass spectra were obtained for the separated peaks shown in **Figure 5.3**. The high quality of the mass spectra facilitate improved library matching exceeding 90% with many having reported matches of 95-99%. An additional benefit of 2GC-MS is the sensitivity enhancement by the effect of modulator, which increases the number of peaks identified in the sample.

A contour plot of 2GC-MS analysis of moderately complex parsley essential oil sample is shown in (**Figure 5.4**). The complementary selectivity is apparent in dimethyl polysiloxane and polyethylene glycol column separations. The non-polar

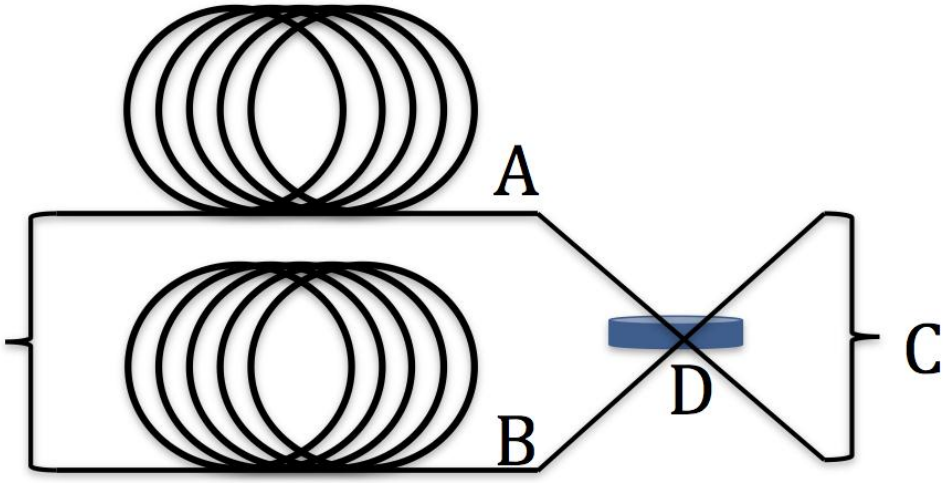

Figure 5.1. Illustration of the column configuration used for dual channel 2GC-MS.

A, polyethylene glycol (*DB-WAX) column*; B, dimethyl polysiloxane *(DB-1) column*; C, MS detector; D, dual stage thermal modulator.

Table 5.1. Advanced modulation parameters for contra-directional modulation. Modulation period 3.0 s with a hot pulse time 1.2 s and cool time between stages 0.3 s.

FIRST STAGE	Cool Time Till Off	Cool Off Time	Heat Time Till On	Heat On Time
	1500 ms	1200 ms	1500 ms	1200 ms
SECOND STAGE	Cool Time Till On	Cool On Time	Heat Time Till Off	Heat Off Time
	1200 ms	1800 ms	1200 ms	1800 ms

column mainly interacts through dispersive forces, whereas the polar column mainly interacts through H-bonding/polarity. Thus 2GC-MS provides two diverse LRIs for each component from two different stationary phases to support mass spectral peak assignments. Qualitative analysis of the separated compounds was performed by MS library searching and *LRI* searching on both columns using Aroma Office 2D software. The Aroma Office 2D software allows selection of specific stationary phases for the literature *LRI* search and comparison. An MS similarity of ≥ 80 % was applied for MS searching. Confirmation of putative identification proceeded by setting Δ*LRI* of ± 10 for the dimethyl polysiloxane stationary phase column and Δ*LRI* of ± 25 for the polyethylene glycol stationary phase column. **Table 5.2** lists peak assignments for identified compounds in parsley essential oil. **Group I** lists a total of 12 peaks confirmed with LRI_1 (non-polar), LRI_2 (polar), MS_1 (non-polar) and MS_2 (polar) searches except apiole (peak 30 LRI: 1633_{obs} and 2438_{obs}). It is well known that this compound is a very important parsley essential constituent. Unfortunately the Aroma Office 2D database doesn't have the LRI information of this compound within the dimethyl polysiloxane (DB-1) and polyethylene glycol (DB-WAX) column search.

Group II lists all other peaks detected in parsley essential oil those at least fail one or two search criteria compared to aforementioned Group I compounds. A total of 12 peaks confirmed by search criteria LRI_1 and MS_1 and these compounds were not seen in the polar column separation. For instance peak 25, germacrene D (peak 25, *LRI* 1401) was positively identified using the non-polar column and the Aroma Office 2D database. With a database *LRI* of 1706 for polar column, this peak must be eluted between peaks 12 and 16 in the polar column. However there is no peak detected in that region (Fig. 5.4). This peak must be co-eluted with some other compounds in the

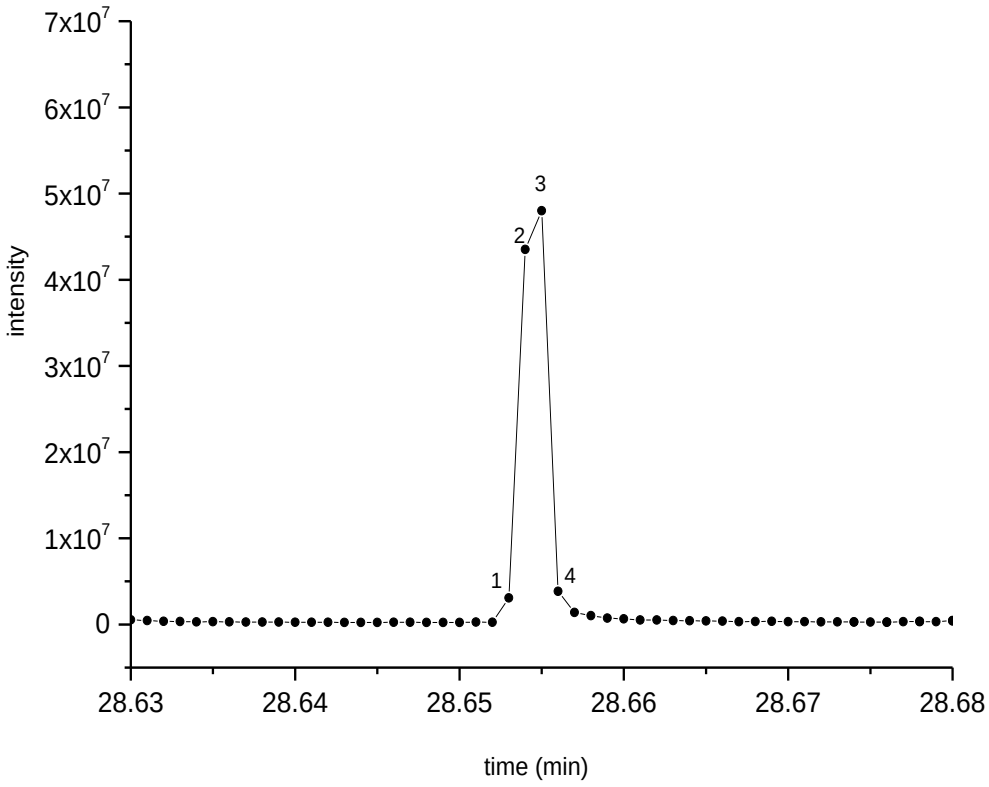

Figure 5.2. Illustration of the four data points for tallest pulse (cal. 28.65) min for peak 26) obtained with dimethyl polysiloxane (DB-1) column.

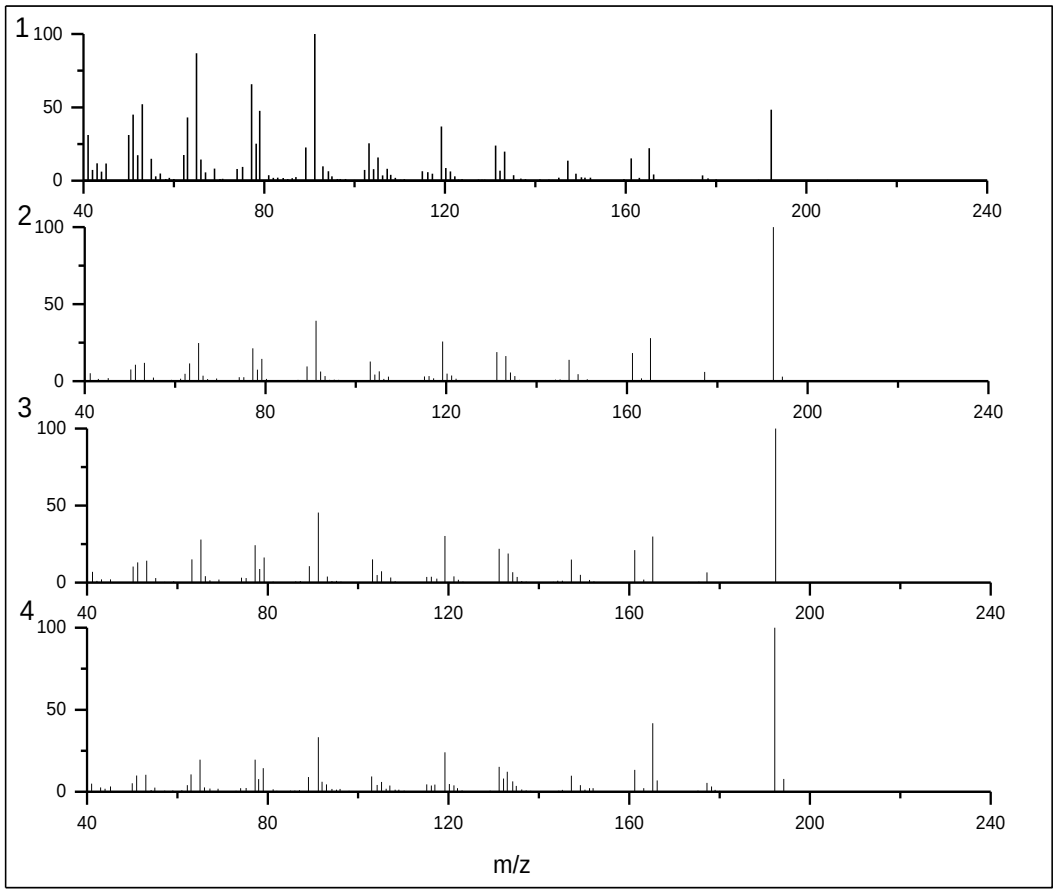

Figure 5.3. Successive mass spectra recorded across the data points (Fig 5.2) (cal. 28.503min) for peak 26 obtained with dimethyl polysiloxane (DB-1) column.

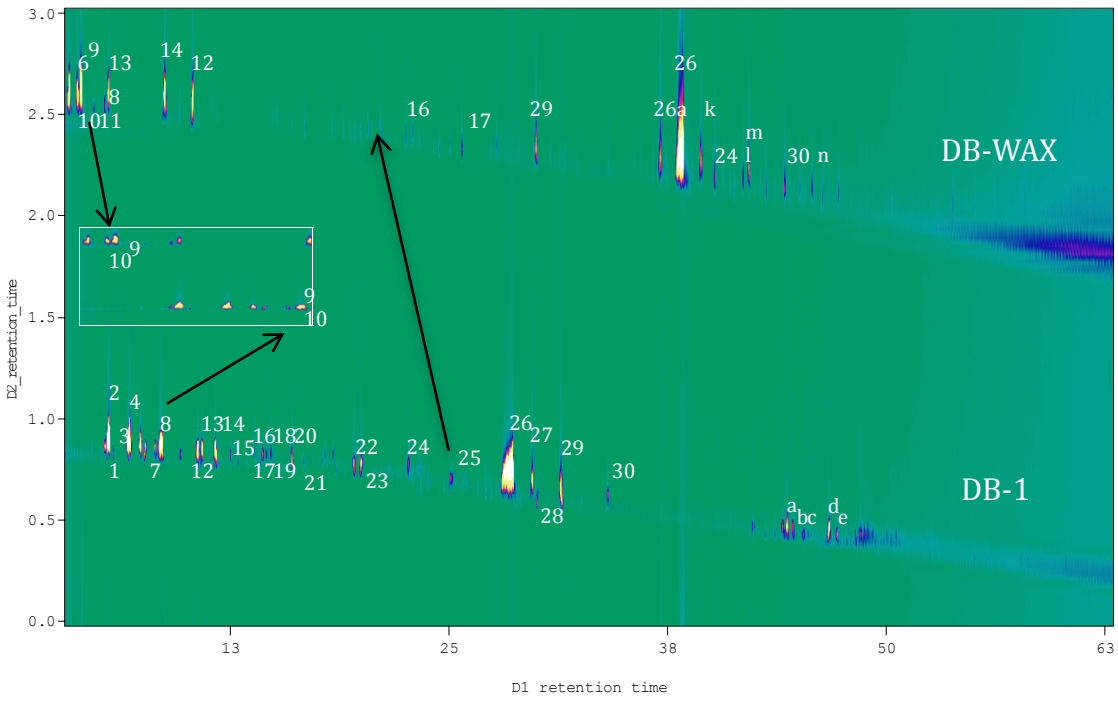

Figure 5.4. Contour plot of parsley essential oil obtained by 2GC-MS system with their respective peak number. Identities of separated components are reported in Table 5.2. The extracted chromatograms with indicator see main text.

Table 5.2. Lists of components identified in the parsley essential oil and their dimethyl polysiloxane (DB-1) and polyethylene glycol (DB-WAX) column linear retention indices (LRIs) with their mass spectral quality.

Peak No	Peak Assignment	DB-1				DB-WAX			
		% match	LRI_{obs}	LRI_{lit}	ΔLRI	% match	LRI_{obs}	LRI_{lit}	ΔLRI
Group I									
6	myrcene	97	984	984	0	97	1166	1167	1
8	*p*-cymene	96	1008	1009	1	98	1256	1266	10
9	β-phellandrene	95	1014	1014	0	97	1229	1216	-13
11	*trans*-β-ocimene	87	1043	1042	-1	90	1238	1251	13
12	α-*p*-dimethylstyrene	99	1068	1066	-2	98	1424	1449	25
13	terpinolene	98	1074	1072	-2	98	1264	1282	18
14	*p*-1,3,8-menthatriene	96	1095	1098	3	96	1376	1402	26
16	p-methyl acetophenone	91	1150	1150	0	88	1771	1791	20
17	*m*-cymen-8-ol	94	1152	1151	-1	90	1849	1825	-24
26	myristicin	95	1491	1493	2	94	2282	2295	13

29	caryophyllene oxide	87	1563	1564	1	86	1998	1997	-1
30	apiole	81	1633	-		81	2498	-	
Group II									
1	α-thujene	93	922	923	1			1022	
2	α-pinene	99	929	929	0			1039	
3	camphene	96	936	936	0			1067	
4	sabinene	99	966	966	0			1130	
5	β-pinene	98	982	983	1			1107	
7	δ-3-carene	91	991	992	1			1145	
10	limonene			1019		96	1193	1201	8
15	unknown		1113						
18	estragole	93	1158	1158	0			1677	
19	unknown		1163						
20	unknown		1190						
21	thymol methyl ether	93	1204	1204	0			1575	
22	unknown		1272						

23	*p*-cymen-7-ol	87	1280	1278	-2			2219	
24	unknown		1344						
25	germacrene D	87	1401	1401	0			1706	
26a	myrsticin artifact					83	2236	2295	59
27	elemicin	89	1522	1525	3			2226	
28	germacrene B	90	1526	1529	3			1810	

polar column. Conversely, the separation of limonene and β-phellandrene (peak 9, *LRI* 1014 and 10, *LRI* 1019) were difficult to separate using the non-polar column due to similar retention behavior in this stationary phase. However these two compounds were well resolved in the polar column. The extracted region of this separation indicated is shown in Fig. (5.4). Previously Poynter and Shellie [147] also reported the co-elution of these two compounds for the analysis of the same essential oil using a high-speed low pressure GC-MS system. The number of compounds noticed using the polar column was less than the number of compounds observed using the non-polar column. This may be due to the co-elution of many parsley essential oil constituents in the polar stationary phase. After cross searching the two *LRI* using the Aroma Office 2D database and following mass spectral matching there were still some peaks unidentified in both columns (Group II, Table 5.2). Mass spectral matching was utilised to confirm some of these peaks. For instance peak 24 (*LRI* 1344) was an unknown compound in the non-polar column and this peak was located in the polar column separation by matching the respective mass spectra shown in **Fig. (5.5**) with peak intensity as an indication. Similarly peak 26a in the polar column was confirmed as an artifact peak of myristicin (peak 26) by matching their respective mass spectra shown in **Fig. (5.6)**.

Several additional unidentified peaks were found in the polar column separation, marked with "k, l, m and n" in Figure 5.4. These peaks belong to a heavier fraction of parsley essential oil and are eluted very late in the non-polar column. The corresponding components are marked with "a, b, c, d, and e" in the polar column separation in Figure 5.4. The calculated LRIs for those compounds are provided in **Table 5.3**. The confirmations of these peaks were performed using mass spectral data and those are shown in **Fig. (5.7)**.

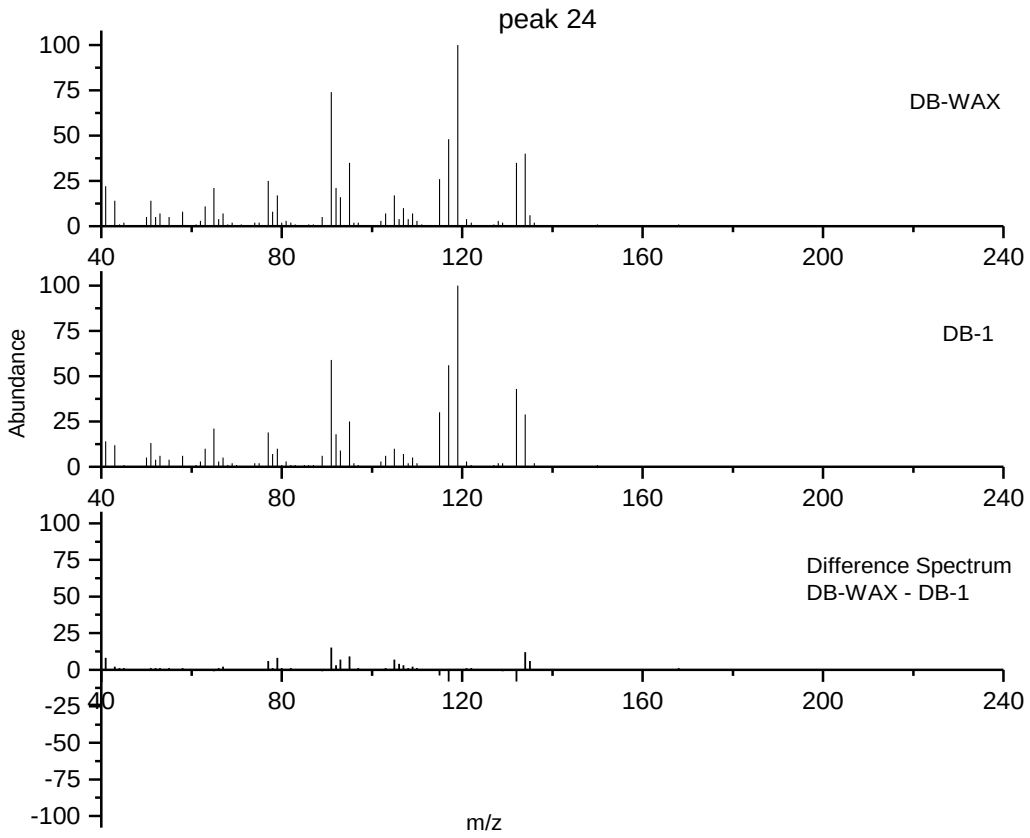

Figure 5.5. MS spectra of peak 24 obtained from both dimethyl polysiloxane (DB-1) and polyethylene glycol (DB-WAX column).

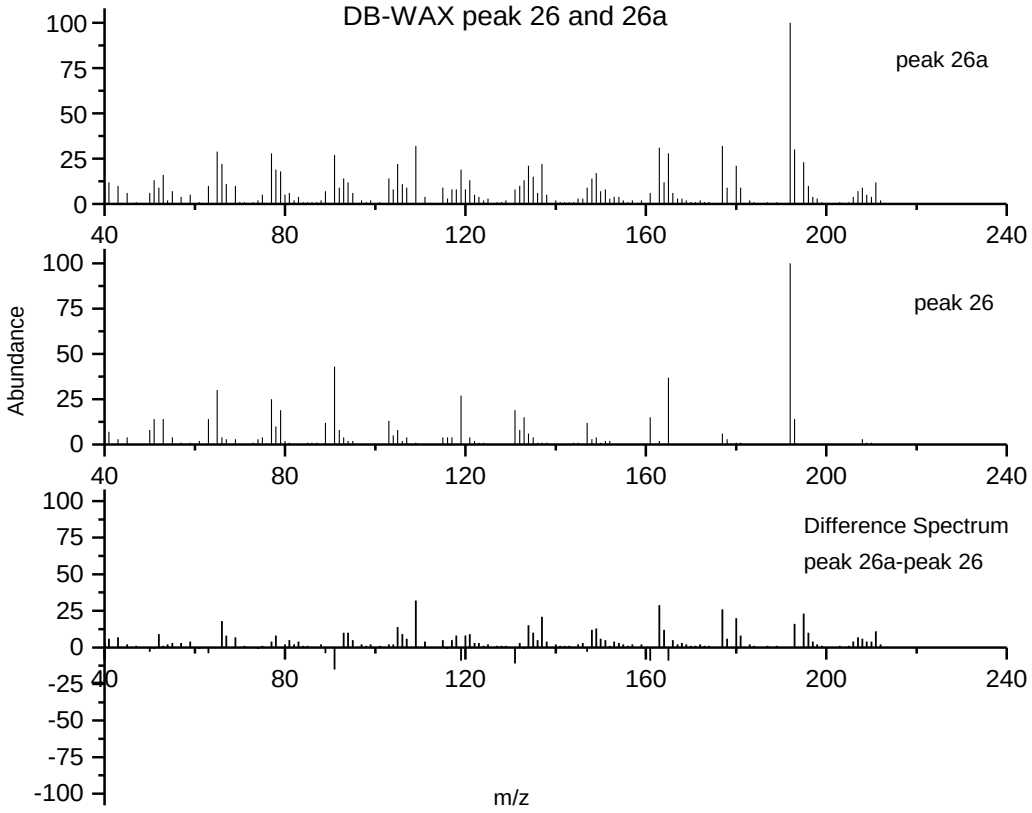

Figure 5.6. MS spectra matching of peak 26 and peak 26a obtained from polyethylene glycol (DB-WAX) column.

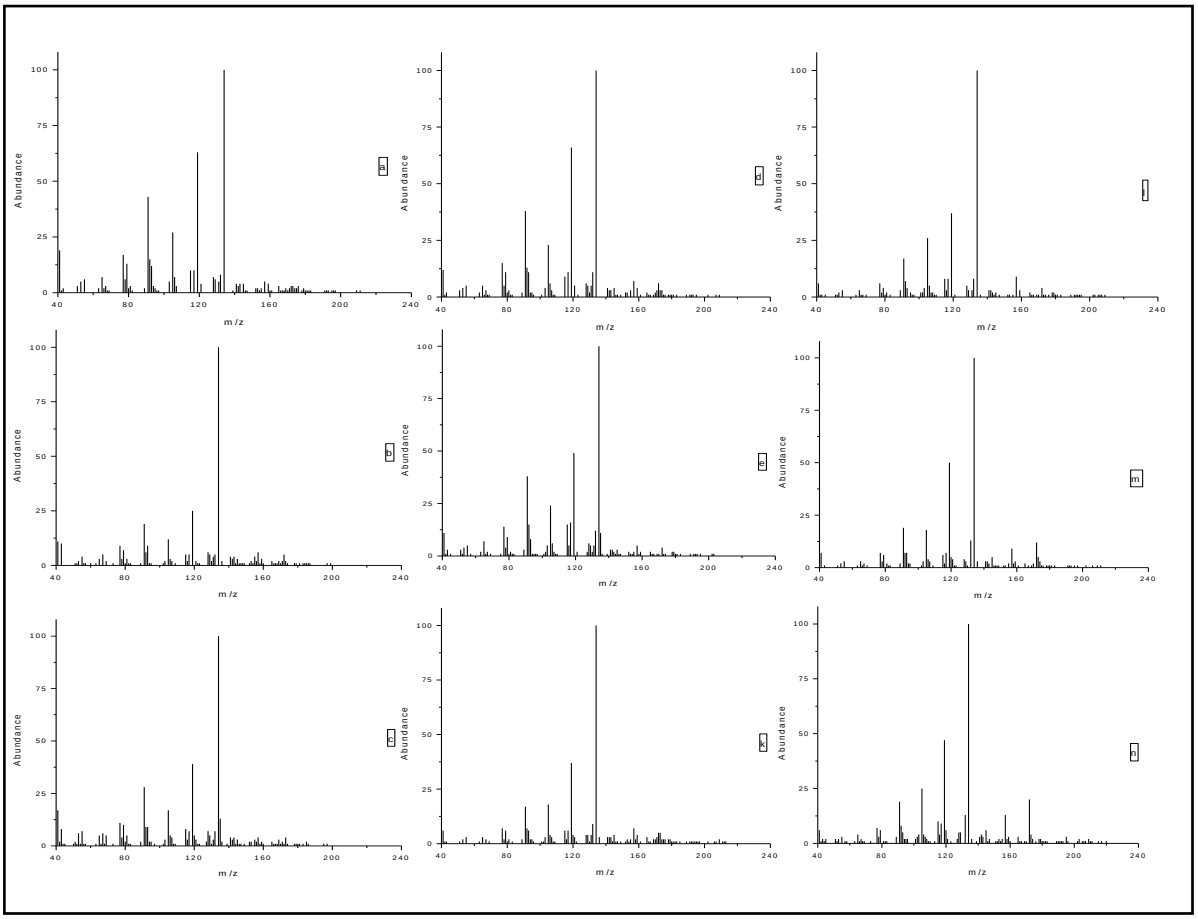

Figure 5.7. MS spectra of 9 peaks. a,b,c,d,e belong to dimethyl polysiloxane (DB-1) column and k,l,m,n belong to polyethylene glycol (DB-WAX) column. See main text for further discussion.

Table 5.3. Retention indices of dimethyl polysiloxane (DB-1) and polyethylene glycol (DB-WAX) unidentified peaks shown in (Fig 6.4).

Peak	LRI (DB-1)	Peak	LRI (DB-WAX)
a	1933	k	2318
b	1943	l	2409
c	1963	m	2420
d	2010	n	2542
e	2025		

5. 4. Conclusion

A dual channel gas chromatography method coupled with mass spectrometry (2GC-MS) was described for the separation and identification of the moderately complex natural product; parsley essential oil. The approach requires only a single detector and provides complementary information about the sample components from two different stationary phases. The method described here is similar to single-dimensional GC methods, and hence expertise for optimisation and analysis is not needed. Once a sample has been characterised, 2GC with FID detection can then used for quantitative analysis of the same sample since the peak pattern in 2GC is reproducible and method translation between MS and FID can aid in establishing conditions where correlation of the two experiments should be reliable. With suitable column ensembles this technique should be applicable to a range of complex samples to overcome many limitations of the commonly used single-dimensional separation.

Chapter 6

Ultra-dimensional separations using multiplexed dual dimensions column comprehensive two-dimensional gas chromatography (2GC × 2GC) with contra-directional thermal modulation

6. 1. Introduction

A multiplexed dual comprehensive two-dimensional gas chromatography (2GC × 2GC) approach designed for complex sample analysis is introduced. The approach produces two comprehensive two-dimensional chromatograms in one run. The two comprehensive two-dimensional chromatograms from this single detector system provide complementary separations by harnessing different selectivity provided by the four separation columns.

To date a vast majority of GC × GC separations have been performed using a non-polar × polar column set, but the advantages of using a reverse column set such as polar × non-polar approach for the analysis of food sample have been reported [148-150]. Essential oils are complex mixtures that often contain several different semi- and highly-polar classes of compounds such as aldehydes, ketones, alcohols and esters. This sample characteristic has been exploited by using different stationary phase columns in one-dimensional GC for essential oil analysis [22,23,151]. Collection of different retention index values on different stationary phases is of critical import for compound identification [22]. As mentioned previously, GC × GC uses different stationary phases in the first- and second-dimension, but second-dimension retention indices are not well established for compound identification, despite some proposed methodologies for retention index calculation[135-138].

Adahchour and co-workers carried out analysis using different types of column combinations in GC × GC [102]. The authors demonstrated the benefits of reverse-ordered structured chromatograms from individual GC × GC analyses for diesel; olive oil and vanilla extract samples. Later the same research group [101] introduced a twin GC × GC instrument configuration using a four-jet two-stage modulator [152] to simultaneously modulate on both separations. In this configuration the sample was split into two first-dimension columns placed parallel and transferred into two second-dimension columns in a single GC oven. The

system produced a pair of independent GC × GC chromatograms from a single run, but used two detectors.

The present investigation builds upon the foundation laid in earlier chapters using the principle of contra-directional modulation. In the present discussion, contra-directional modulation permits an instrument configuration that generates two independent GC × GC chromatograms, using four different stationary phases in a single run without recourse to dual injectors or dual detectors. A combination of polar × non-polar and non-polar × polar column sets was used for the separation of parsley and hop essential oils. The complementary selectivity and future opportunities this enabling instrumentation affords are highlighted.

6. 2. Experimental

Samples and sample preparation

Steam distilled parsley essential oil was provided by Essential Oils of Tasmania (Kingston, Australia). Hydrodistilled hop essential oil was provided by Hop Products Australia (Bellerive, Australia). Both oils were diluted tenfold in dichloromethane (Sigma-Aldrich) prior to analysis.

Instrumentation

2GC × 2*GC-FID*

All analyses were performed using a Leco GC × GC-FID instrument with LN2 Cooled Thermal Modulator (Leco Australia). The chromatograph was equipped with split/splitless injector, operated at 230 ^{o}C in split mode throughout (with 20:1 split ratio). Hydrogen carrier gas was generated using a Parker Balston H2PEM-260 hydrogen generator. 2GC × 2GC analyses were performed by splitting the flow from the injected sample into two first-dimension columns by means of a twin-hole graphite ferrule (SGE Analytical Science), all

other column connections were made using press tight connectors (Restek). The first dimension columns were installed contra-directionally in the GC × GC modulator. The two first-dimension columns were a 30 m × 250 μm i.d. fused silica capillary coated with 0.25 μm dimethyl polysiloxane (DB-1; Agilent Technologies) and a 30 m × 250 μm i.d. fused silica capillary coated with 0.25 μm polyethylene glycol (DB-WAX; Agilent Technologies). The two second-dimension columns were a 0.5 m × 0.10 mm i.d. × 0.10 μm film thickness coated with 5% Phenyl 95% dimethyl polysiloxane stationary phase and 0.5 m × 0.10 mm i.d. × 0.10 μm film thickness coated with polyethylene glycol stationary phase. Both second-dimension columns were from Restek. Total carrier gas flow rate was 1.2 mL/min (Hydrogen; constant flow). The oven temperature program was 40 ^{o}C (0.2 min hold) to 220 ^{o}C (3 min hold) at 7 ^{o}C/min. Flow from the two second-dimension columns was directly passed into a single detector by means of twin hole graphite ferrule. For convenience of column installation and operation, the auxiliary (second-dimension) column oven was removed from the GC × GC system. All four columns were heated using the main GC oven. The modulation period and its timing parameters are described in Results and Discussion. The modulator offset temperature was 15 ^{o}C. Effluent from each secondary column was monitored by a single flame-ionisation detector. The data acquisition rate and the operating temperature were 100 Hz and 250 ^{o}C respectively. Data were collected and summarized using Leco ChromaTOF software.

6. 3. Results and discussion

Two comprehensive two-dimensional chromatograms are achieved from a single injection using the instrument configuration shown in **Figure 6.1**. First-dimension column (A) and second-dimension column (C) produce one two-dimensional chromatogram and first-dimension column (B) and second-dimension column (D) produce the other two-dimensional chromatogram without peaks from either separation interfering with peaks from the other.

Like previous chapters, two independent GC × GC chromatograms can be acquired using a single detector, and viewed in a single chromatogram window.

By selecting quite different column sets, each of the separations are expected to be different even though the separation was carried under a single operational condition. To demonstrate this, parsley and hop essential oils were analysed and the multiplexed two-dimensional chromatograms are shown in **Figure 6.2** and **Figure 6.3** respectively. In the top half of Figures 6.2 and 6.3 (*dimethyl polysiloxane* × *polyethylene glycol* column combination) a strong interaction with the second-dimension column stationary phase increases retention in the second dimension column. The separations presented in the bottom half of Figures 6.2 and 6.3, which represent a column combination of polyethylene glycol × 5 % phenyl 95% dimethyl polysiloxane, demonstrate how a strong interaction with the first-dimension column stationary phase decreases retention in the second-dimension. Previously Adahchour and co-workers demonstrated similar retention behaviour of non-polar × polar and polar × low-polar column set for the separation of fatty acids methyl esters (FAME's), marine sediment extract [101] and food samples [102]. Although the present instrumentation provides two primary and two secondary retention times for a single analytes no attempt was made to assign identity to any of the separated components. Based on the work presented in Chapter 4 and 5 it is entirely feasible that coupling with MS would facilitate further sample characterisation. The method described here is amenable to coupling with MS without any further modification of the instrumental configuration.

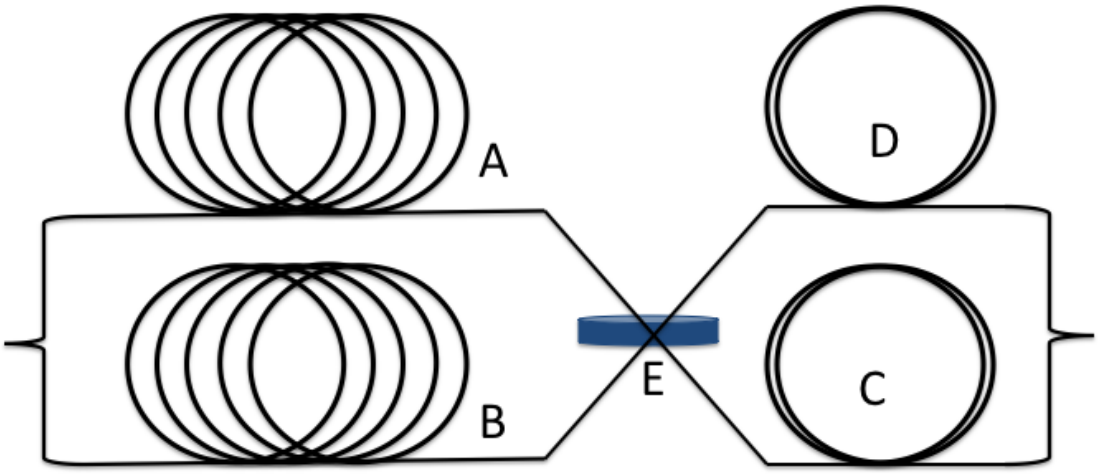

Figure 6.1. Illustration of the column configuration used for multiplexed dual 2GC × 2GC

(A) polyethylene glycol; (B) dimethyl polysiloxane; (C) 5% phenyl 95% dimethyl polysiloxane; (D) polyethylene glycol; (E) Dual stage thermal modulator.

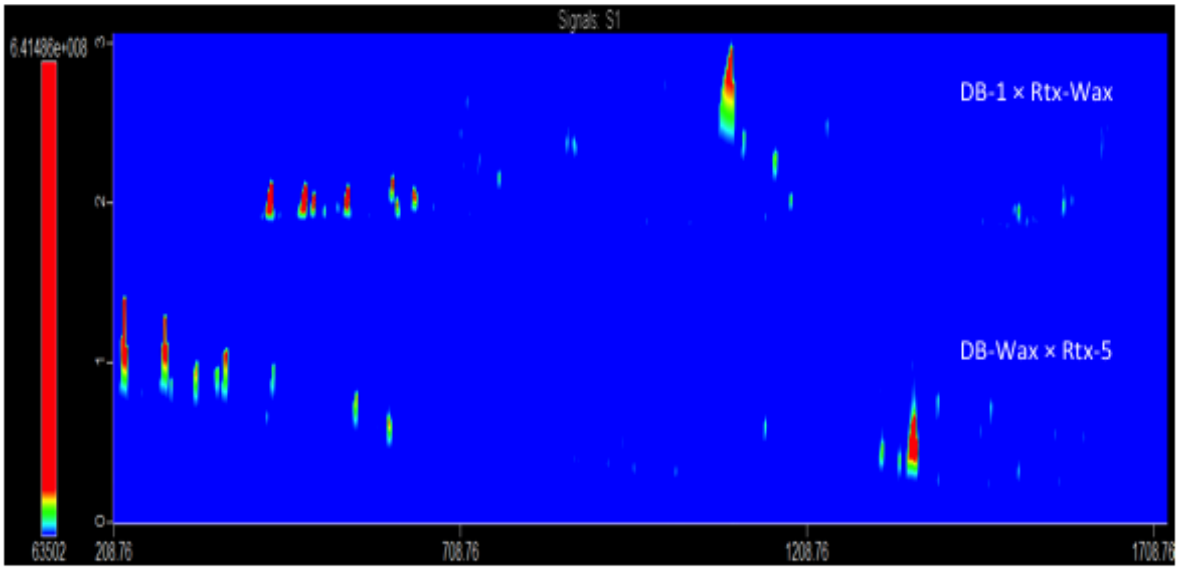

Figure 6.2. Multiplexed two-dimensional separation space for the separation of parsley essential obtained using 2GC × 2GC.

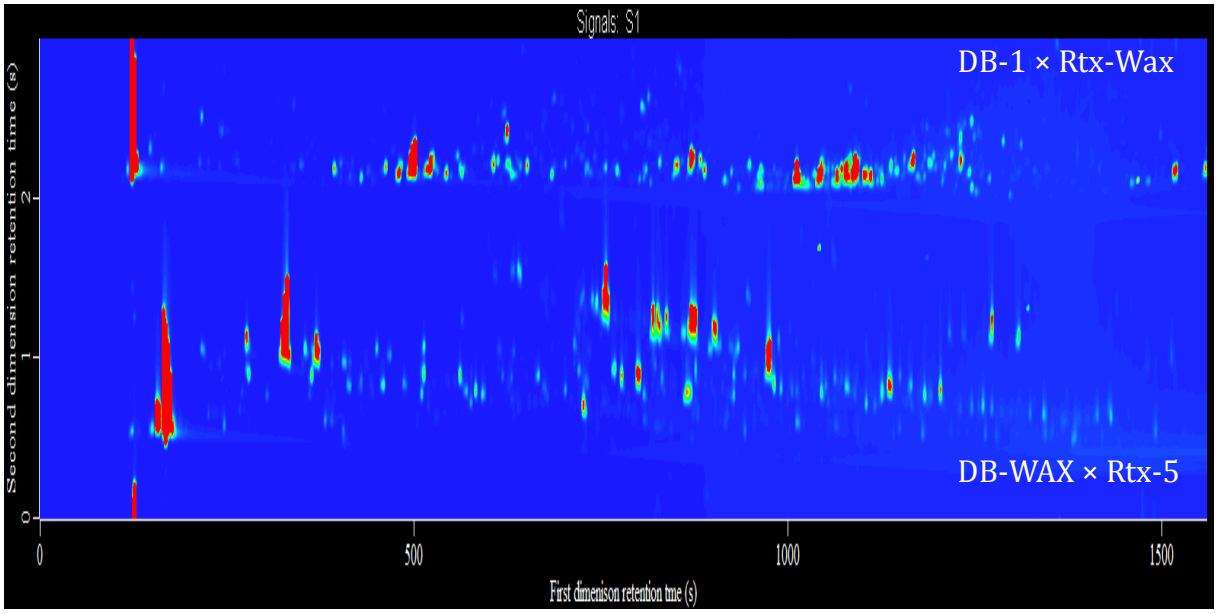

Figure 6.3. Multiplexed two-dimensional separation space for the separation of hop essential obtained using 2GC × 2GC.

6. 4. Conclusion

Multiplexed 2(GC × GC) is highly promising for qualitative analysis, since it provides four sets of independent retention times (or retention indices) in a single analysis. The present study unleashes the full potential of contra-directional modulation; two GC × GC chromatograms are obtained from an essentially standard commercial GC × GC system leading to an appreciable productivity gain. The present study employed column ensembles often used in the essential oil analysis. It is possible that other column configurations can be developed to extend the scope of 2(GC × GC) analysis for various complex mixtures, keeping in mind that particular attention need be paid while developing and implementing any new operating conditions to minimise detrimental wraparound effects.

Coupling 2(GC × GC) to mass spectrometry will provide further strength of the approach described here. Combination of four retention times (or retention indices) with mass spectrometry should provide unequivocal assignment of individual peak identity in multicomponent samples.

Chapter 7

Conclusions and Further Research

7. 1. Conclusion

The ultimate aim of any high resolution GC analysis is to obtain maximum information about sample components within the shortest possible timeframe. This thesis discussed the development of a series of high-resolution GC techniques for the separation of complex mixtures, particularly plant extracts. Throughout the thesis there was an emphasis on increasing analysis productivity, either by way of maximising kinetic performance of one-dimensional separations, or by capitalising on the contra-directional modulation concept to permit multiplexed GC operation.

Investigation of highly efficient narrow bore column under speed optimised flow conditions was performed on one-dimensional GC in Chapter 2. The investigation explored possibilities for maximal kinetic performance available from a maximum inlet pressure of 100 psi (689 kPa) generated from a commercially available gas generator. Both polar and non-polar columns provided peak capacity higher than 1000, with a 40 m long narrow bore column. Efficiency optimised flow conditions led to only marginal peak capacity improvement compared to analysis under speed optimised flow conditions. The analysis of *T. lanceolata* plant extract showed that speed optimised flow conditions provided similar efficiency as optimised flow conditions, but within a 30% reduced analysis time.

A new contra-directional modulation regime using a dual stage modulator was introduced in Chapter 3. This chapter explored the concept of GC × 2GC using a single detector system. Contra-directional modulation experiments were performed using a column configuration with two second-dimension columns. The result obtained for the standard mixture was similar to previously published GC × 2GC approaches that utilised multiple detectors. Additional stationary phase selectivity is always beneficial in complex mixtures separation, and achieving this in a single run is beneficial in terms of time and cost. The only concern of

this GC × 2GC approach is proper tuning of two second-dimension columns with GC operating conditions, since wraparound effect is highly detrimental and must be avoided.

A new approach called "multiplexed dual first-dimension comprehensive GC × GC combining with MS (2GC × GC-MS)" approach was introduced in Chapter 4. Unlike GC × 2GC, in the 2GC × GC study contra-directional modulation was performed on the two first-dimension columns. The approach is highly beneficial for essential oil industries, which require multiple retention indices with mass spectral information for component identification. In this study a polar and non-polar column in the first-dimension and a mid-polar column in the second-dimension were chosen. The two first-dimension columns with a one second-dimension column provided two concurrent GC × GC chromatograms from a single injection, with single detection. The analysis of *M. alternifolia* tea tree essential oil using 2GC × GC-MS with Aroma Office 2D database facilitated unequivocal peak assignment of separated peaks. The 2GC × GC approach separated the complex mixtures into very pure components prior to the MS detection, improving overall the characterisation of sample components. The only concern of 2GC × GC is the second-dimension column which is carefully optimised to avoid the so-called "wraparound" effect due to the single detector operation.

Chapter 5 discussed the concept of dual channel GC using a single detector system. This was a serendipitous outcome from development of contra-directional modulation in this research program. Dual channel GC uses a single detector system and it eliminates the requirements of multiple detectors. Parsley essential oil analysis was performed to prove the principle of 2GC-MS instrumentation. Unequivocal peak assignments were carried out using mass spectral information and two retention indices. Combining the Aroma Office 2D database with the current 2GC-MS instrumentation makes data analysis easy. Unlike other multiplexed approaches described above in this thesis, this approach requires minimal optimisation of

operational parameters. One could choose any column dimensions or operational parameter to perform the analysis. Multiplexed dual channel 2GC-MS is a powerful method for reliable characterisation of sample components when high sample throughput is required.

Chapter 6 described the design and implementation of a multiplexed dual first and second-dimension column comprehensive two-dimensional gas chromatography system 2(GC × GC) using a single detector. This provided separation of analytes through four different separation columns. Contra-directional modulation was performed on the two first-dimension columns, although, there was possibility of performing contra-directional modulation two second-dimension columns. This type of instrumentation provided two sets of two-dimensional chromatograms like the aforementioned multiplexed GC approaches, but separations were achieved from four different stationary phases, further enhancing the separation power. The analyses of parsley and hop essential oils were performed using 2(GC × GC) instrumentation. The separation behaviour was similar to hitherto reported twin GC × GC instrumentation that utilised multiple detectors.

7. 2. Further research

This research introduced a new concept of contra-directional modulation using a commercially available thermal modulator and has led to the development of a series of novel multidimensional GC techniques. Each approach developed herein using a single detector system provided highly satisfactory results and so is a promising field for further research. Although the present research program showed examples of 2GC × GC-MS and 2GC-MS instrumentation with a scanning (quadrupole) mass spectrometer, translation to time-of-flight mass spectrometry with high-speed acquisition will avail full potential of the multiplexed GC approaches introduced herein, and permit validation of the approaches thus far presented as

proof-of-principle. 2GC-TOFMS has perhaps the great potential application in many fields since it does not restrict any operating conditions or column selection.

Selection of appropriate stationary phases for broader specific applications will provide further justification of multiplexed GC approaches. These approaches are applicable to petrochemicals, forensic science, environmental and biological samples. There is no single column set available for GC × GC at the time of writing to separate all individual 209 PCBs mixtures in the literature. It is anticipated that one could select a column combination of DB-1 × BPX-50 and DB-XLB × VF-23ms for the separation of PCB mixtures using 2GC × 2GC-TOFMS instrument.

GC × 2GC may be used for GC × GC separation and GC × *enantio*-GC separation in a single run. Calculating retention indices in the second-dimension separation will offer very rich information when applied to GC × 2GC-TOFMS and 2(GC × GC)-TOFMS, and improve the characterisation of sample components. This study effectively illustrated the benefit of multiplexed dual column GC approaches with Aroma Office 2D retention index database. This provides a powerful identification strategy for essential oil analysis.

For ease of installation and to ensure robustness it is recommend that a twin-hole modulator be developed to maintain alignment of the two capillaries in the GC × GC modulator system. In the present Leco GC × GC system, utilised in this study, the secondary oven mounted just above the modulator makes it difficult to connect two column in contra-directional mode. Expanding the oven space with appropriate design of a secondary oven will make for an easiest column installation and optimisation of method development for chromatographers. Furthermore, the availability of appropriate software for data analysis including the Aroma Office 2D database will also be beneficial for the multiplexed approaches described in this

study. The above recommendations are worthy of consideration to manufactures dealing with instruments and procedures described in this thesis.

All of the studies in this thesis were performed using a Leco modulator. One of the main areas of research to be undertaken regards the performance of the contra-directional modulation using alternative modulator available to date. Two physically separated modulation stages are essential for performing contra-directional modulation. Quad-jet, dual-jet, LMCS, and loop-type systems are all amenable to contra-directional operation, although the latter design may require modification to allow installation of two modulator loops, and is probably quite difficult to optimise. The present investigation developed the contra-directional modulation using liquid N_2 modulator that may restrict uptake for many users due to the expense of operation. It is anticipated that utility of less expensive liquid CO_2 modulator or other commercial available consumable-free GC × GC modulators such as closed-cycle refrigerant or immersion cooling systems can be utilised for this process.

Further opportunity also exists to explore multiplexed GC approaches using pneumatic modulation systems, for instance differential flow modulator. Use of pneumatic modulation systems for multiplexed GC will at least require proper tuning of column configuration and optimisation of operating conditions. It is noteworthy that additional steps may be required to couple them with mass spectrometry since pneumatic modulators typically operate using high second-dimension flow rates.

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Appendix

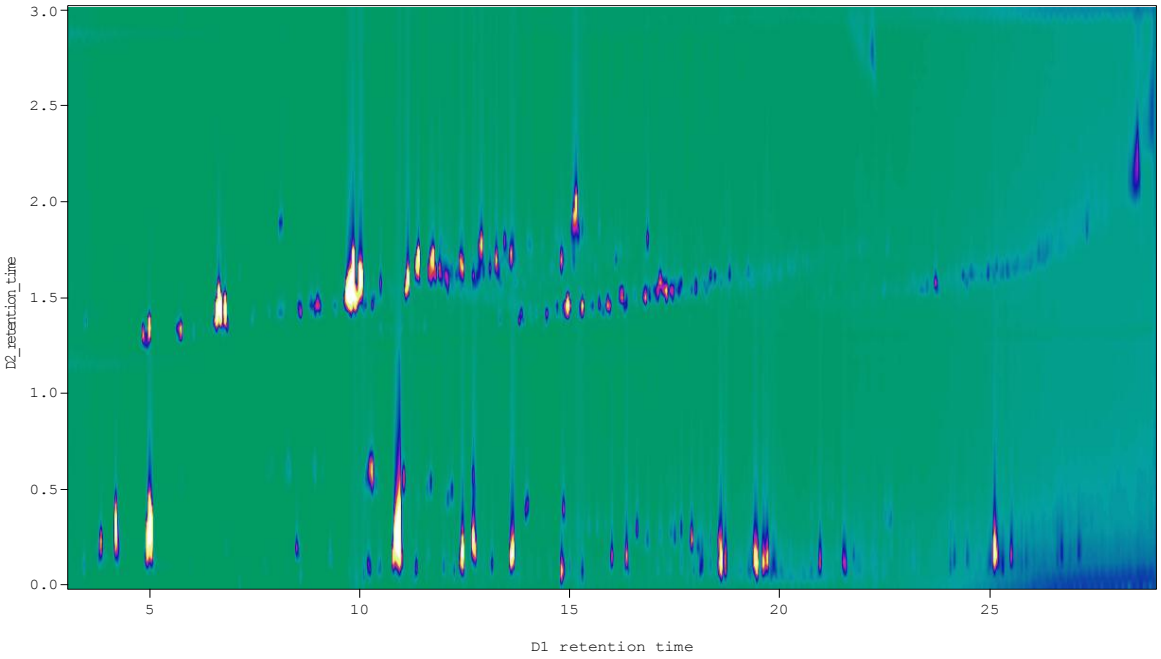

Figure A1. 2GC × GC-MS chromatogram of Australian tea tree essential oil. Retention times in the second-dimension column differ from 2GC × GC-FID results due to modulation phase error caused by modulation start time inconsistency brought about by employing a solvent-delay in the MS experiments.

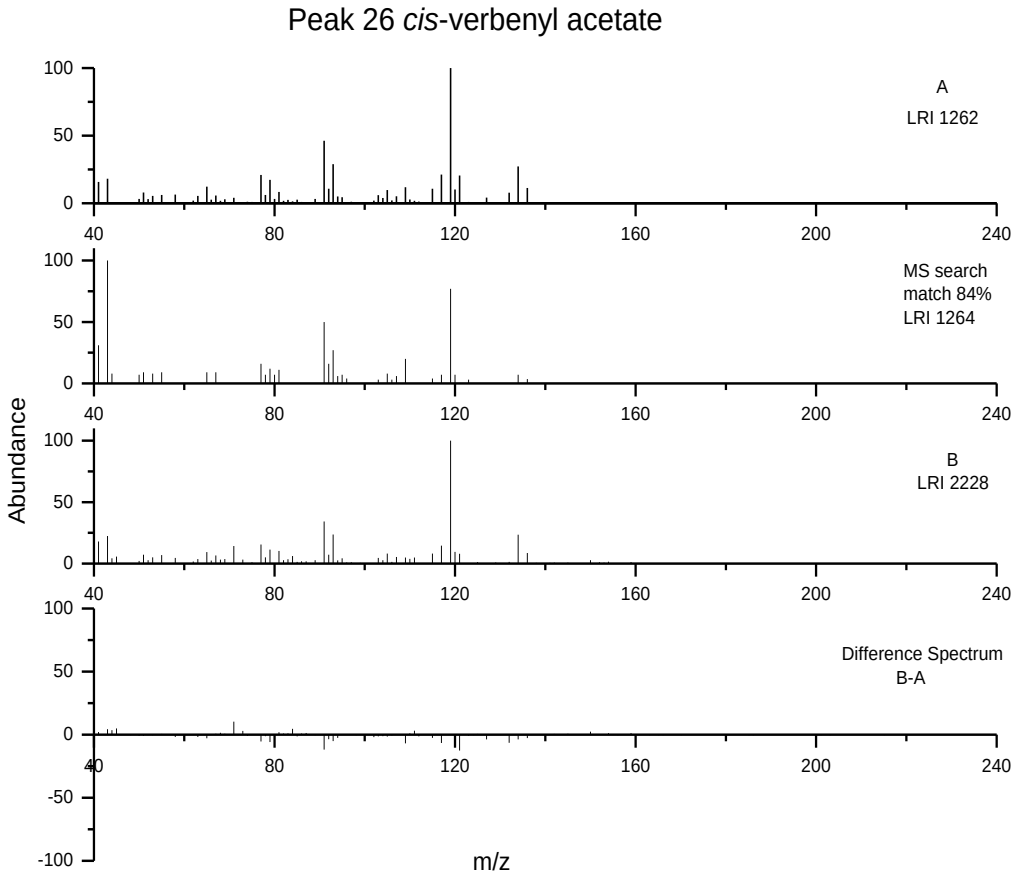

Figure A2. Matching of MS spectra of peak 26 *cis*-verbenyl acetate (Table 4.3) with MS library search match spectrum. (A) polar × mid-polar, (B) polar × mid-polar.

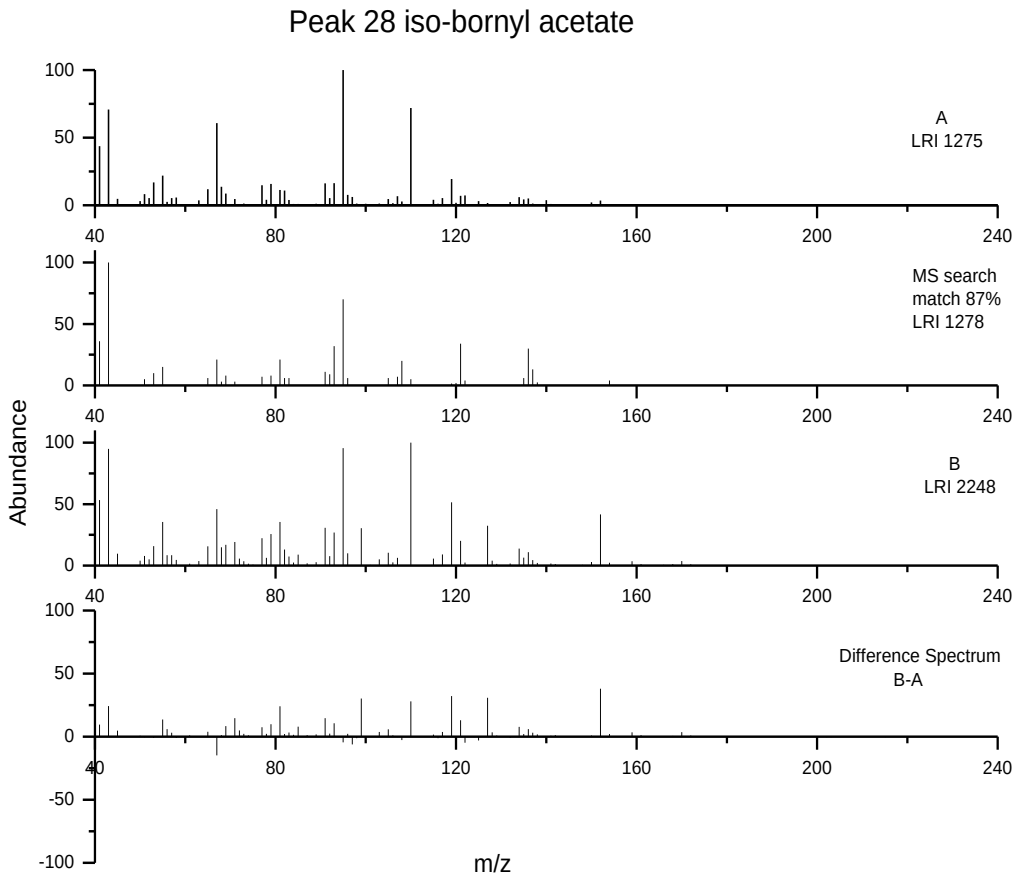

Figure A3. Matching of MS spectra of peak 28 iso-bornyl acetate (Table 4.3) with MS library search match spectrum. (A) polar × mid-polar, (B) polar × mid-polar.

.

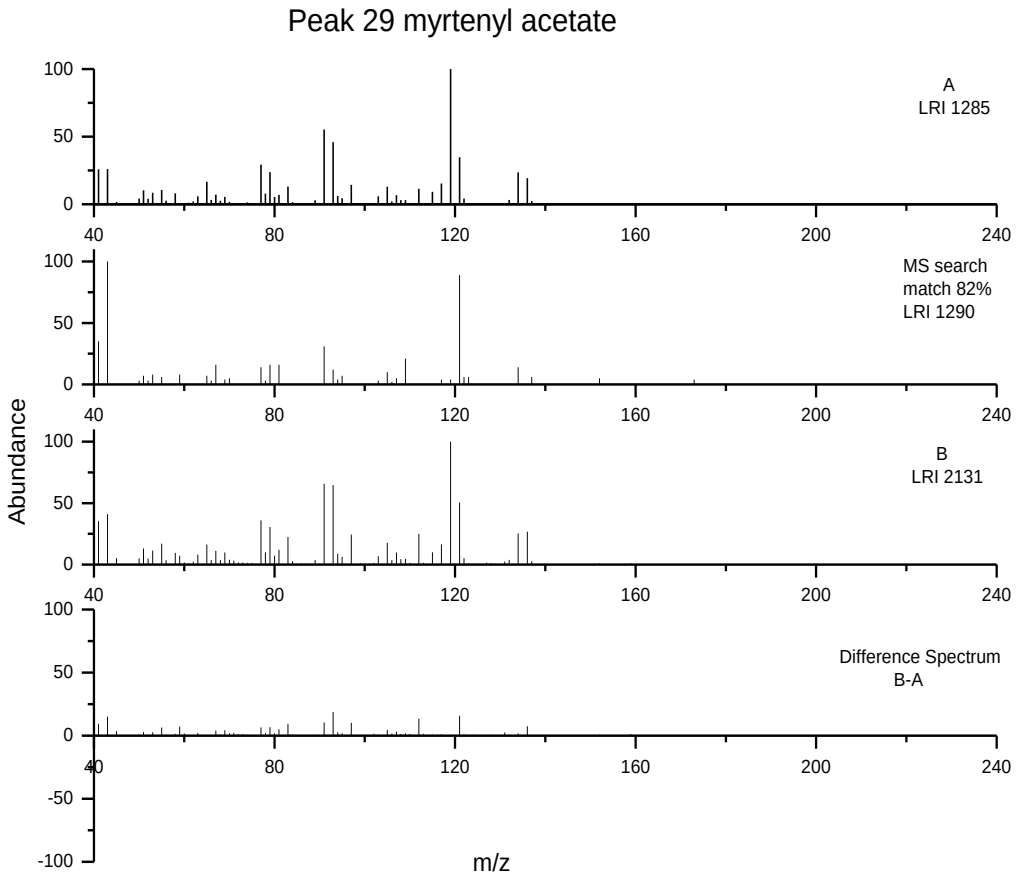

Figure A4. Matching of MS spectra of peak 29 myrtenyl acetate (Table 4.3) with MS library search match spectrum. (A) polar × mid-polar, (B) polar × mid-polar.

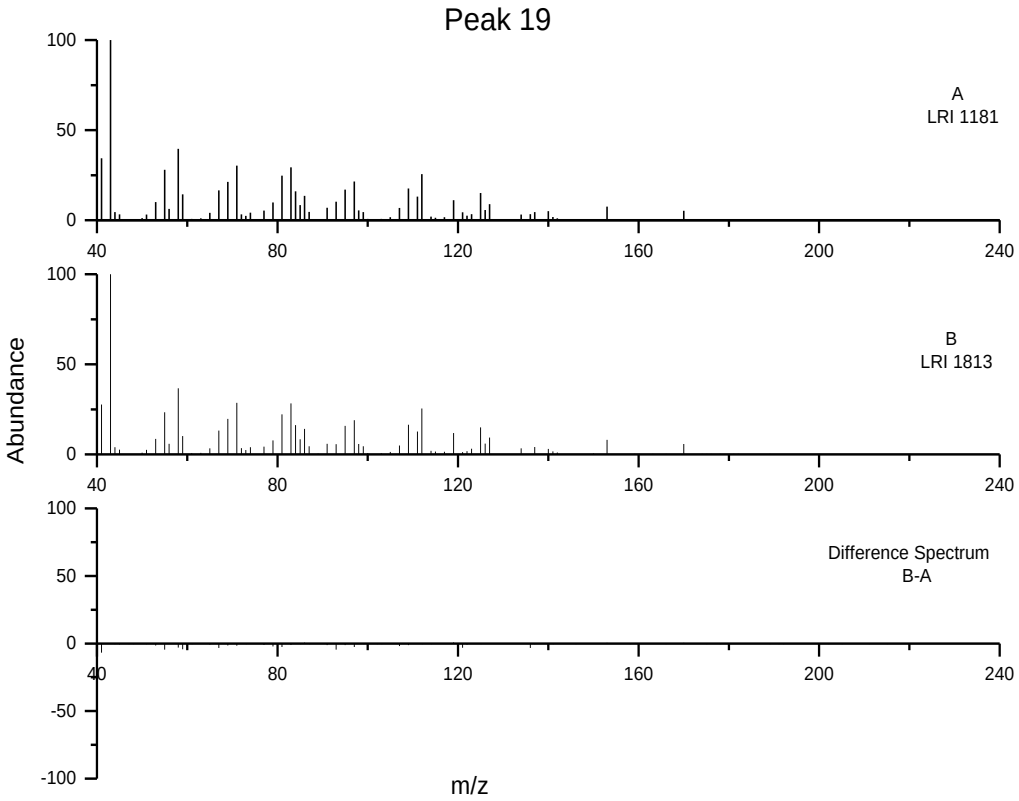

Figure A5. Matching of MS spectra of peak 19 (Table 4.3). (A) polar × mid-polar, (B) polar × mid-polar.

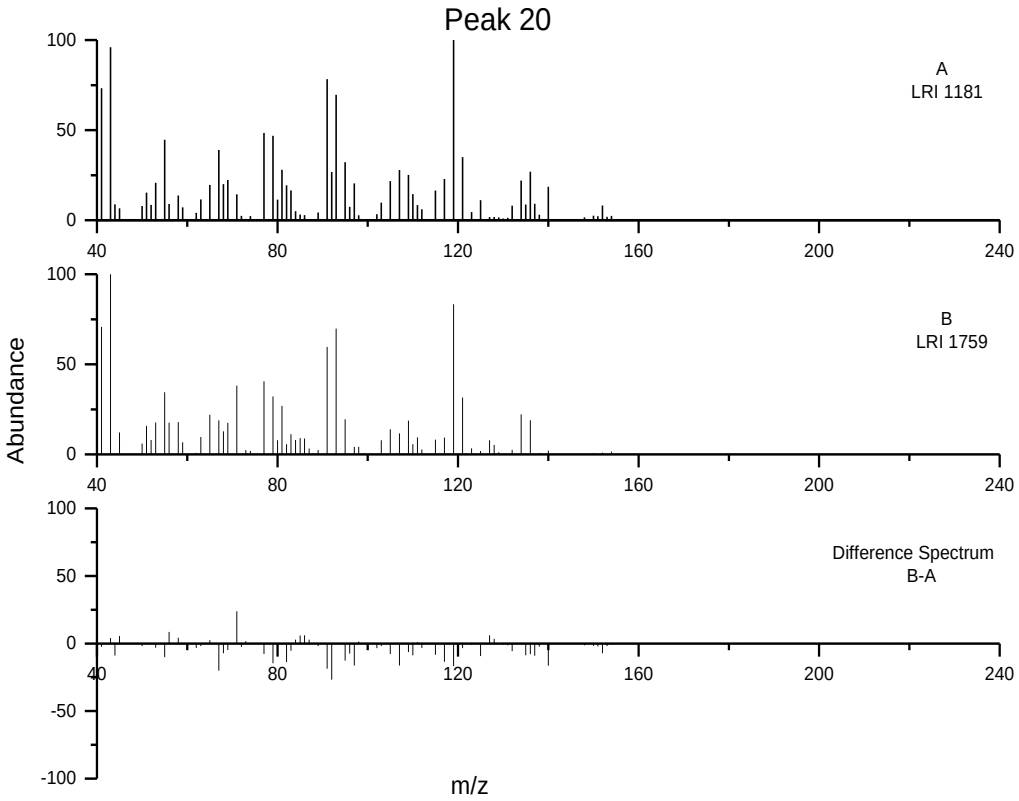

Figure A6. Matching of MS spectra of peak 20 (Table 4.3). (A) polar × mid-polar, (B) polar × mid-polar.

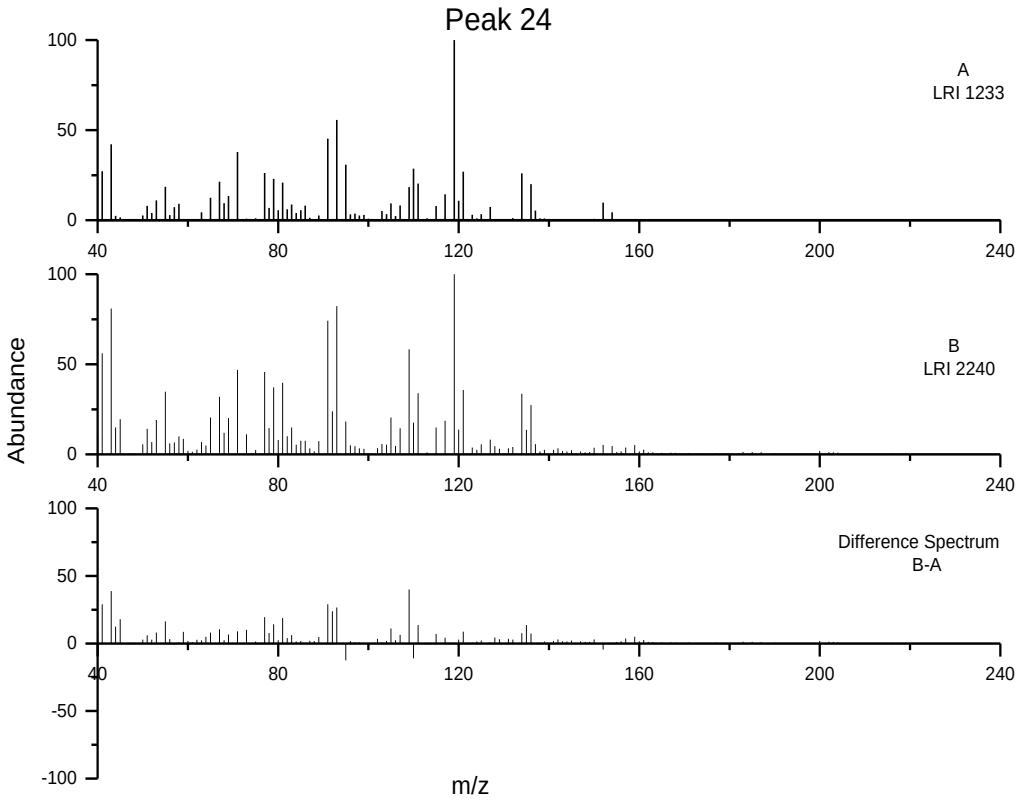

Figure A7. Matching of MS spectra of peak 24 (Table 4.3). (A) polar × mid-polar, (B) polar × mid-polar.

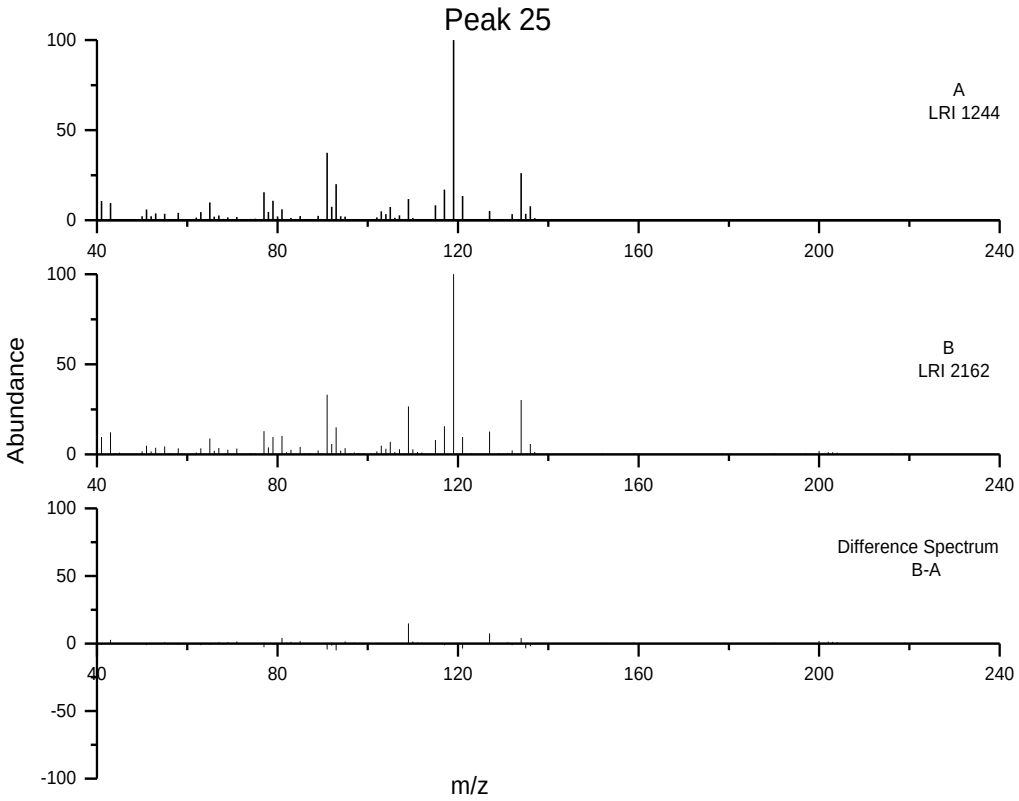

Figure A8. Matching of MS spectra of peak 25 (Table 4.3). (A) polar × mid-polar, (B) polar × mid-polar.

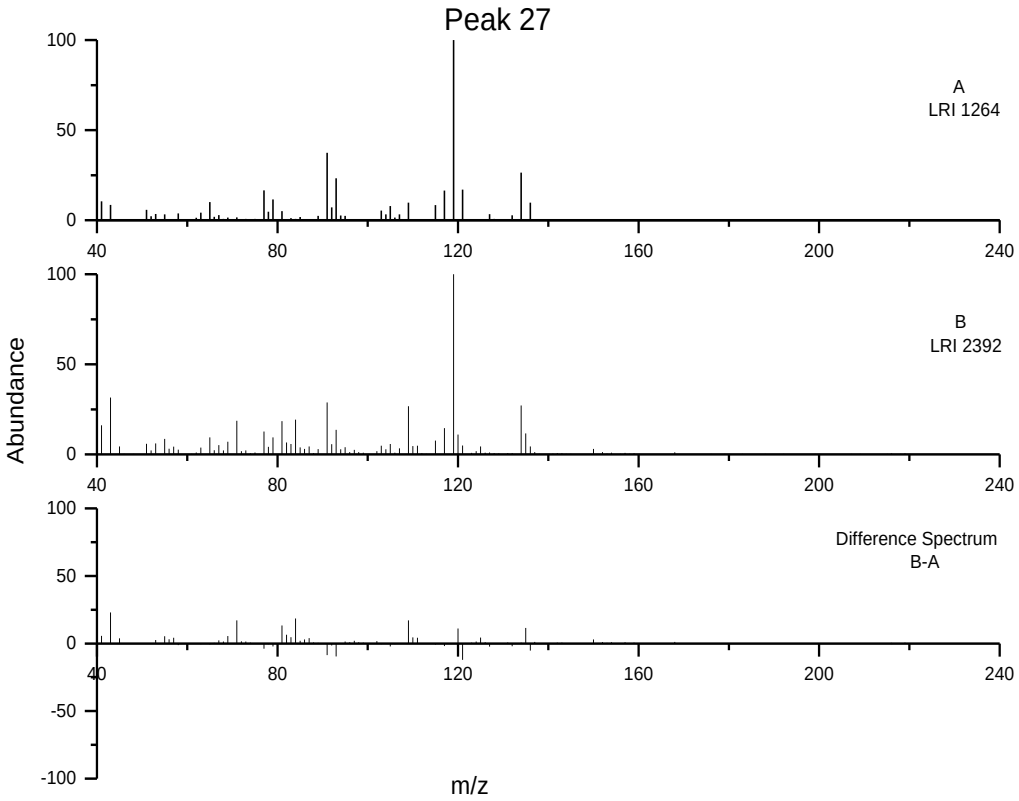

Figure A9. Matching of MS spectra of peak 27 (Table 4.3). (A) polar × mid-polar, (B) polar × mid-polar.

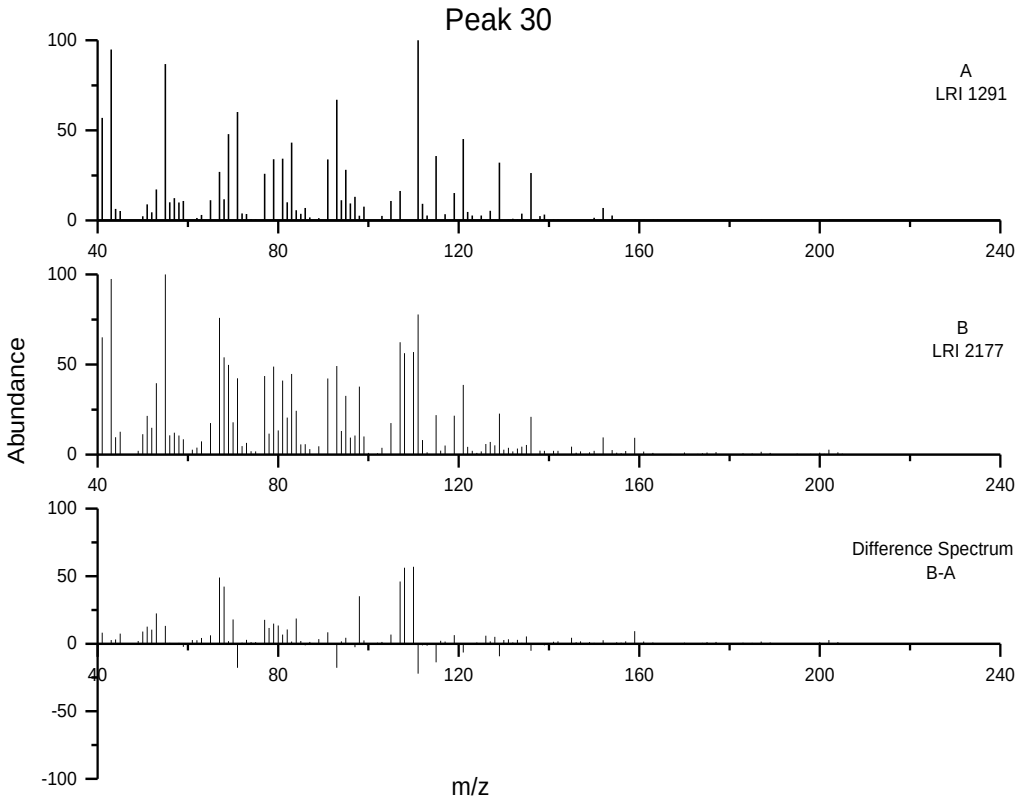

Figure A10. Matching of MS spectra of peak 30 (Table 4.3). (A) polar × mid-polar, (B) polar × mid-polar.

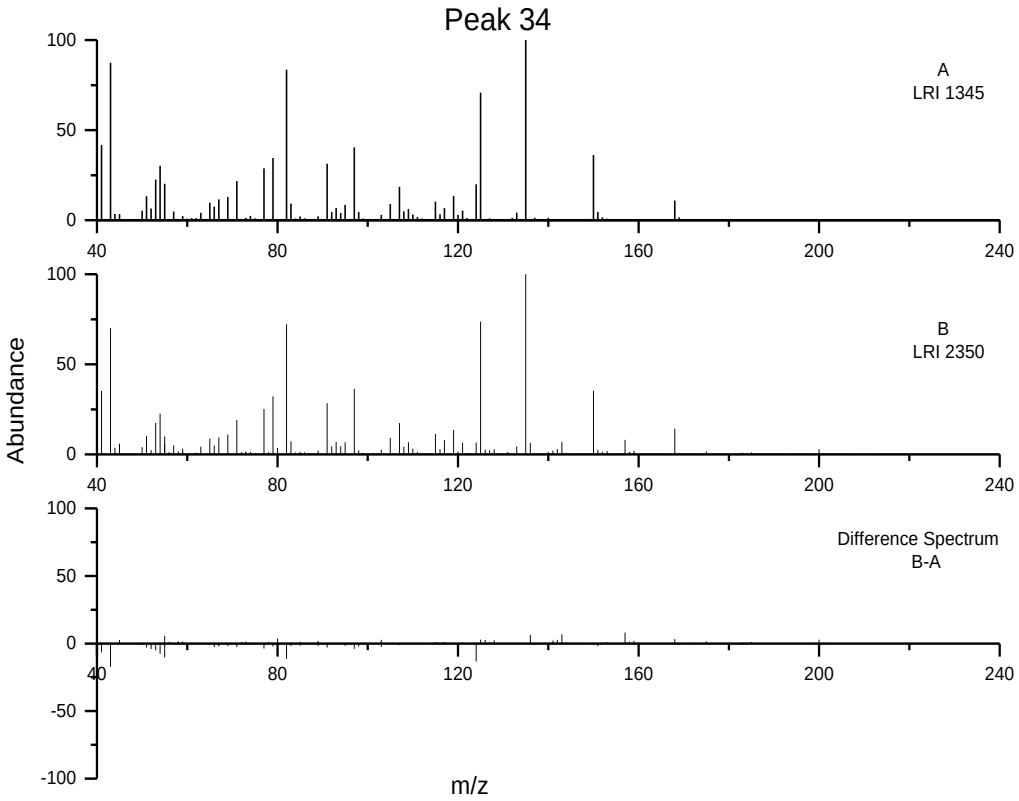

Figure A11. Matching of MS spectra of peak 34 (Table 4.3). (A) polar × mid-polar, (B) polar × mid-polar.

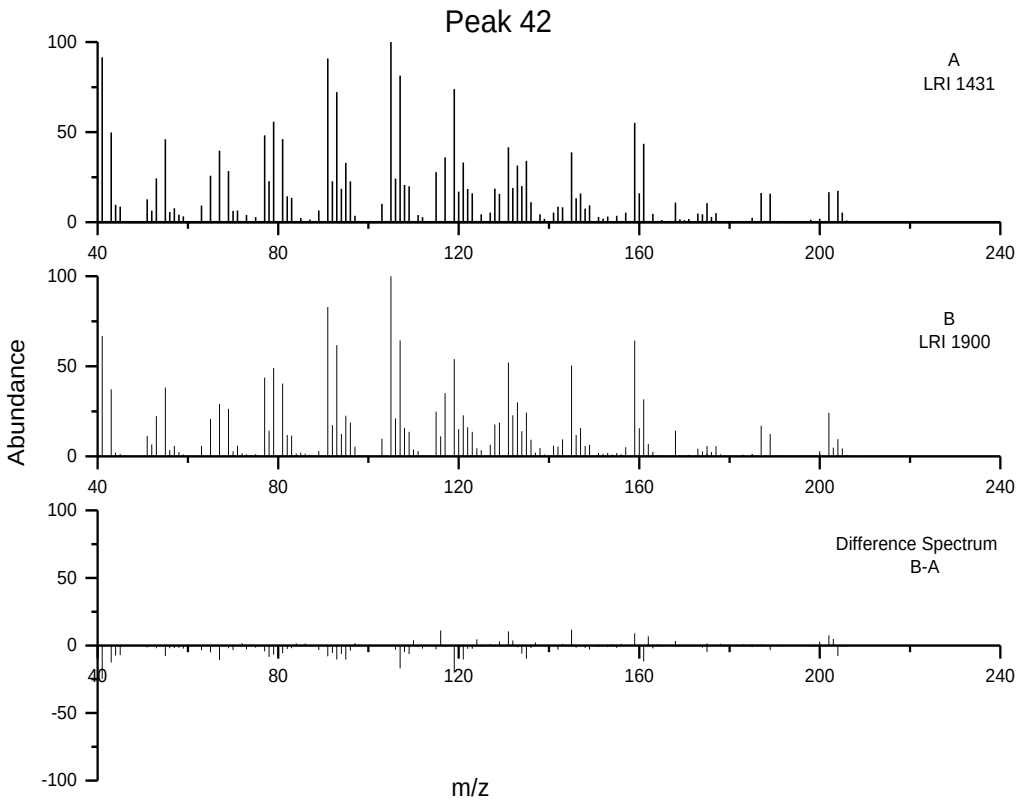

Figure A12. Matching of MS spectra of peak 42 (Table 4.3). (A) polar × mid-polar, (B) polar × mid-polar.

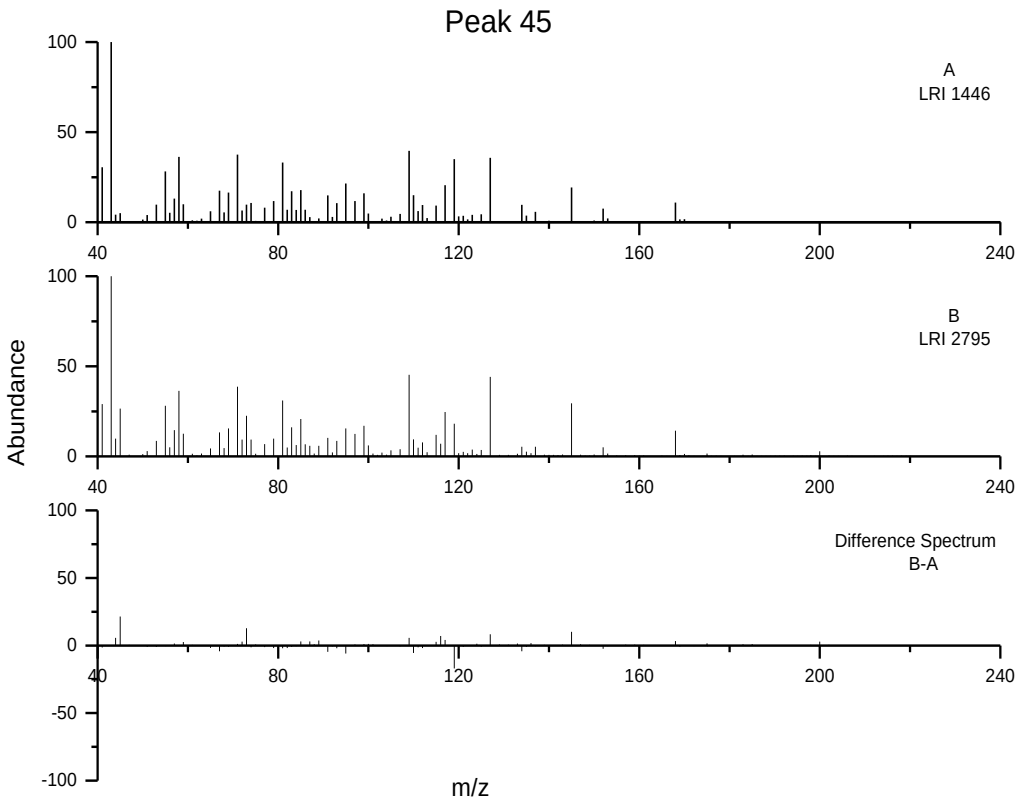

Figure A13. Matching of MS spectra of peak 45 (Table 4.3). (A) polar × mid-polar, (B) polar × mid-polar.