Application of comprehensive two-dimensional gas chromatography for the characterisation of the volatile composition of Honeybush tea

by

Gaalebalwe Elizabeth Ntlhokwe

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Supervisor: Prof. André J. de Villiers
Co-supervisor: Dr. Andreas G.J. Tredoux

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Summary

Honeybush tea (*Cyclopia* spp.), like many other natural products, contains a large number of volatile compounds with different physiochemical properties and present at widely varying concentrations. This presents a significant analytical challenge for conventional one-dimensional gas chromatography (GC). The aim of this study was to explore the potential of comprehensive two-dimensional gas chromatography (GC×GC) for the improved analysis of honeybush tea volatile compounds.

The first part of the study focused on the evaluation of a GC×GC system equipped with a novel single-stage thermal modulator. A chromatographic method well suited for the analysis of honeybush volatiles was successfully developed by systematic optimisation of instrumental parameters. Solid phase micro-extraction (SPME) was used for extraction of volatile compounds prior to GC×GC separation. Three *Cyclopia* species were analysed: *C*. *genistoides*, *C*. *subternata* and *C*. *maculata*. Chromatographic separation of honeybush volatiles was significantly improved on this system. 69 compounds were identified based on the retention data obtained by injection of reference standards in combination with flame ionisation detection (FID). Hyphenation to quadrupole mass spectrometry (qMS) was also explored, although the slow acquisition rate of the detector provided limited scope for identification of additional compounds. The highly reproducible performance of this modulator proved beneficial in the use of multivariate data analysis of the GC×GC-FID data. Significant differences in the volatile composition between the three *Cyclopia* species were observed, in general agreement with sensory studies for the same set of samples.

In the second part of the study, a commercial GC×GC instrument equipped with a cryogenic modulator and hyphenated to time-of-flight MS (TOF-MS) was used for the detailed qualitative analysis of the same set of samples. This configuration provided new insight into the volatile composition of honeybush tea: the combination of enhanced GC×GC separation and sensitivity, coupled to the high-speed acquisition of TOF-MS allowed the tentative identification of 158 compounds in these samples for the first time. A total of 272 compounds were identified using either reference standards or mass spectral and retention index (RI) data. Significant differences in the volatile composition of *C. genistoides*, C*. subternata* and *C. maculata* were also observed in the GC×GC-TOF-MS data. (*E*)-cinnamaldehyde was identified in honeybush tea for the first time, only in *C. maculata* samples. This compound is likely responsible for the characteristic aroma of this species identified by sensory analysis.

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Opsomming

Heuningbostee (*Cyclopia* spp.), soos wat ook die geval is vir talle ander natuurprodukte, bevat ‘n goot aantal vlugtige komponente met baie diverse fisiochemiese eienskappe en wat voorkom in ‘n groot verskeidenheid van verskillende konsentrasies. Dit bied ‘n groot analitiese uitdaging in terme van die gebruik van konvensionele een-dimensionele gaschromatografie (GC). Die doel van hierdie ondersoek was om die potensiaal van twee-dimensionele gaschromatografie (GC×GC) vir die verbeterde analise van heuningbostee te ontgin.

Die eerste deel van hierdie studie fokus op die evaluasie van ‘n GC×GC sisteem wat toegerus is met ‘n nuut-ontwikkelde enkelfase termiese modulator. ‘n Chromatografiese metode, geskik vir die analise van vlugtige komponente in heuningbostee, is met sukses ontwikkel deur die sistematiese optimalisering van instrumentele instellings. ‘Solid phase micro-extration’ (SPME) is aangewend vir die ekstraksie van die vlugtige komponente alvorens die GC×GC analise uitgevoer is. Drie *Cyclopia* spesies is geanaliseer: *C*. *genistoides*, *C*. *subternata* en *C*. *maculata*. Die chromatografiese skeiding van vlugtige verbindings in heuningbostee is beduidend verbeter deur gebruik te maak van hierdie sisteem. 69 komponente is geïdentifiseer, gebaseer op die retensie data wat verkry is met die inspuit van verwysing standaarde en in kombinasie met vlam ionisasie deteksie (flame ionisation detection, FID). Koppeling met ‘n kwadrupool massaspektrometer (qMS) is ook ondersoek maar die stadige data-opneemspoed het die identifikasie van nuwe komponente belemmer. Die baie goeie vlak van herhaalbaarheid wat hierdie modulator bied was veral voordelig m.b.t. die gebruik van multi-veranderlike dataprosessering. Beduidende verskille in die samestelling van vlugtige komponente tussen die drie *Cyclopia* spesies is gevind en stem in die algemeen ooreen met sensoriese studies wat op dieselfde monsters uitgevoer is.

In die tweede deel van hierdie studie is ‘n kommersieel beskikbare GC×GC sisteem, toegerus met ‘n ‘kriogeniese’ modulator en gekoppel aan ‘n ‘vlugtyd’ MS (TOF-MS) gebruik vir die in-diepte, kwalitatiewe analise van dieselfde stel monsters. Hierdie opstelling het tot nuwe insig met betrekking tot die samestelling van vlugtige verbindings in heuningbostee gelei: die kombinasie van die verbeterde skeiding en sensitiwiteit, tesame met die vinnige data-opneemspoed het die voorlopige identifikasie van 158 nuwe komponente moontlik gemaak. ‘n Totaal van 272 is geïdentifiseer met behulp van óf standaarde, óf massaspektrometrie en retensie indeks (RI) data. Beduidende verskille is ook hier in die vlugtige komponente se samestelling van *C. genistoides*, C*. subternata* en *C. maculate* waargeneem in die GC×GC-TOF-MS inligting. (*E*)-kaneelaldehied is vir eerste keer in heuningbostee geïdentifiseer, en ook slégs in *C. maculata*. Hierdie verbinding speel moontlik ‘n rol in die unieke aroma van hierdie tee, soos wat deur ‘n sensoriese ondersoek aangetoon is.

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List of abbreviations

AED	:	Atomic emission detector
amu	:	Atomic mass units
ANOVA	:	Analysis of variance
CAR	:	Carboxen
cm/sec	:	Centimetre per second
csv	:	Comma separated values
CI	:	Chemical ionisation
DA	:	Discriminant analysis
DC	:	Direct current
DCM	:	Dichloromethane
d_f	:	Column film thickness
DSA	:	Descriptive sensory analysis
DVB	:	Divinylbenzene
EI	:	Electron impact
ECD	:	Electron capture detector
EM	:	Electron multiplier
FID	:	Flame ionisation detector
GC	:	Gas chromatography
GC×GC	:	Comprehensive two- dimensional gas chromatography
GC-MS	:	Gas chromatography-mass spectrometry
GC-O	:	Gas chromatography-olfactometry
GLC	:	Gas-liquid chromatography
GSC	:	Gas-solid chromatography
HPLC	:	High performance liquid chromatography
HS-SPME	:	Head space solid phase micro-extraction

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Hz	:	Hertz
i.d.	:	Internal diameter
IS	:	Internal standard
kPa	:	Kilo Pascal
LLE	:	Liquid-liquid extraction
LMCS	:	Longitudinally modulated cryogenic system
LOD	:	Limit of detection
LOQ	:	Limit of quantification
min	:	Minute
mg	:	Milligram
mL	:	Millilitre
MPDD	:	Miniaturized pulsed discharge detector
MS	:	Mass spectrometry
m/z	:	mass to charge ratio
MSD	:	Mass spectrometric detector
MDGC	:	Multidimensional gas chromatography
NCD	:	Nitrogen chemiluminescence detector
NPD	:	Nitrogen phosphorus detector
NIST	:	National Institute of Standards and Technology (US Department of Commerce)
OTTs	:	Open tubular traps
PA	:	Polyacrylate
PCA	:	Principle component analysis
PDMS	:	Polydimethylsiloxane
PEG	:	Polyethylene glycol
ppm	:	Parts per million
PTFE	:	Polytetrafluroethylene

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PTV	:	Programmed temperature vaporisation (injector)
qMS	:	quadrupole Mass spectrometer
RAM	:	Random access memory
RF	:	Radio frequency
RI	:	Retention index
rpm	:	Revolutions per minute
RT	:	Retention time
RSD	:	Relative standard deviation
SCD	:	Sulphur chemiluminescence detector
SDE	:	Simultaneous steam distillation/extraction
SEP	:	Sample enrichment probe
SCD	:	Sulphur chemiluminescence detector
S/N	:	Signal-to-noise
SPE	:	Solid phase extraction
SPME	:	Solid phase micro-extraction
TIC	:	Total ion chromatogram
TOF-MS	:	Time-of-flight mass spectrometry
t_R	:	Retention time
$\bar{u}$	:	Linear velocity
V	:	Voltage

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This thesis is represented as a compilation of manuscripts not yet submitted for publication. Each manuscript is a chapter of an individual entity and some repetition between chapters has, therefore, been unavoidable.

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Chapter 1

General introduction and Objectives

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1.1. General introduction

Globally tea is the second most consumed beverage after water. Tea is produced from plant materials which grow mostly in the tropical and subtropical regions [1]. For many years teas have been enjoyed mainly for pleasure. However, with many health problems faced by humanity today there is increasing interest in the use of natural products to aid in general health and hence corresponding interest in the health promoting properties of teas [2]. While volatiles *per se* have not been implicated as health promoting compounds, they play a vital role in the pleasant aroma of tea and therefore quality, consumer preference and market value. Production of the tea involves picking of the leaves and stems of the tea plant followed by various processes such as steaming, rolling, fermentation and drying, all of which have an effect on the quality of tea by ultimately affecting the chemical composition. Other factors such as climate, geographical origin, and pre- and post-harvest treatment also affect the tea quality hence standardised production methods have been developed [3,4].

Normally the quality of tea is assessed by human sensory analysis which encompasses properties like aroma, taste and appearance. Although sensory analysis is the primary method used for quality control of food and beverages analytical chemistry techniques such as liquid chromatography (LC), capillary electrophoresis (CE) and gas chromatography (GC) are increasingly being used for chemical characterisation of tea, also for quality control purposes and for detection of adulteration [5].

Honeybush tea is an indigenous South African herbal tea. It is prepared from the shrubs of *Cyclopia* species (spp.) which are endemic to the South African fynbos biome and grow in the coastal region of the Western and Eastern Cape. Herbal tea constitutes the highest economical value of honeybush, although the product also has potential uses in the nutraceutical and cosmetic industries due to unique phenolic content [6]. Of the 23 *Cyclopia* spp. that have been described, mainly three, i.e. *C. genistoides*, *C subternata* and *C. intermedia* are being used for commercial purposes. Currently *C. genistoides* and *C. subternata* are cultivated, whereas *C. intermedia* which contributes a major part of annual production is almost exclusively wild-harvested since cultivation is not favoured [6]. The honeybush industry is still relatively small, especially compared to rooibos tea industry, but is rapidly expanding. This has elicited interest in using other species such as *C. maculata*, *C. sessiliflora* and *C. longifolia* for production. Most products that are available in the local market comprise of a mixture of several honeybush species, and the sensory properties of the individual species are not taken into account when the species are mixed. Since the chemical composition of the plant is liable to vary between species as a function of geographical origin, and climate, failure to take these factors into account, results in honeybush tea of variable and inconsistent quality.

For this reason, a sensory wheel has recently been developed to be used as a quality control tool for honeybush tea [7]. The study included species which are currently used for commercialisation *C. genistoides*, *C. subternata* and *C. intermedia*, and *C. maculata*, *C. longifolia* and *C. sessiliflora* which are been considered for commercial cultivation. The characteristic sensory profile of honeybush tea was established and the differences in sensory properties between species were elucidated. The species were classified into three groups

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according to their sensory characteristics: group A (*C. sessiliflora*, *C. intermedia* and *C. genistoides*) associated with “fynbos-floral”, “fynbos-sweet” and “plant-like” attributes, group B (*C. longifolia* and *C. subternata*) with “rose geranium” and “fruity-sweet” and group C (*C. maculata*) with “woody”, “boiled syrup” and “cassia/cinnamon”. The spicy cassia/cinnamon aroma which distinguished *C. maculata* from the other species has not been used to describe honeybush before; this elicited interest in which aroma-active compound(s) is/are responsible for this note. Gas chromatography-olfactometry (GC-O) was used to identify eugenol which has a spicy character although a compound directly associated with cassia/cinnamon aroma was not identified [7].

Aroma has been shown to be the one of the most important sensory properties of honeybush tea. The perceived aroma is related to the composition of (semi-) volatile compounds in the sample, which is therefore of interest in honeybush research. To date, however, relatively few studies on the volatile composition have been reported. Le Roux and co-workers reported an extensive study of the volatile profiles of the honeybush tea species *C. genistoides*, *C. intermedia*, *C. subternata*, and *C. sessiliflora* studied by gas chromatography-mass spectrometry (GC-MS) and GC-O [8,9]. A total of 255 volatile compounds were identified, 46 of which were detected as aroma-active compounds [10].

Gas chromatography (GC) is the most widely used technique for the analysis of volatile compounds. Despite the proven performance of the technique, however it is often not possible to completely separate the large number of volatile components present in complex samples like honeybush tea in a single analysis. One way to improve the separation is to use multidimensional chromatography, where more than one separation step is used to improve the separation. Comprehensive two-dimensional gas chromatography (GC×GC) is one such technique which utilises two orthogonal GC separations to separate volatile components in a single analysis. The components of the sample are separated on the first column, trapped by the interface (called a modulator) and then injected into the second column for further separation according to different mechanism(s) [11].

Hyphenation of GC to mass spectrometry (MS) provides a powerful tool for structural elucidation and identification of separated compounds. GC×GC coupled to MS provides a ‘third’ dimension where volatile compounds can be identified. Quadrupole mass spectrometers (qMS) are most widely available, but the duty cycle of these instruments is typically too slow for hyphenation to GC×GC analysis. From this perspective, time-of-flight mass spectrometry (TOF-MS) is ideally compatible with GC×GC, and the hyphenated technique GC×GC-TOF-MS has found extensive application in the analysis of highly complex samples [12].

The utility of GC×GC has been demonstrated for the analysis of volatile components in numerous foods and beverages [13,14]. However, one of the biggest drawbacks of the technique, which limits its more widespread application, is the high running costs of most commercially available GC×GC instrumentation. This is largely due to the operation of cryogenic modulators, which require large amounts of expensive cryogenic agents. Several alternative modulators have been designed, including valve-based, pneumatic and thermal

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modulators [15]. Each of these has particular advantages and disadvantages in terms of cost and performance.

Within this context, the aim of this study was to develop GC×GC methods for the improved analysis of honeybush tea volatiles. Two instruments were used in this study: (i) a prototype cost-effective single-stage thermal modular developed by Górecki's group [16,17] and (ii) a commercial GC×GC system utilising cryogenic modulation and coupled to TOF-MS. The aims and objectives of the study are outlined below.

1.2. Aims and objectives

The aim of this study was to develop comprehensive two-dimensional gas chromatography (GC×GC) methods to improve the separation of honeybush tea volatile compounds. Three *Cyclopia* spp. *C. genistoides*, *C. subternata*, and *C. maculata* were studied and the following objectives were devised to achieve the goal of the study:

i) The first part of the study was to develop GC×GC method using a GC×GC system retrofitted with a novel single-stage thermal modulator and coupled to flame ionization detector (FID) and quadrupole mass spectrometry (qMS). This involved

- Optimisation of sample preparation technique and this was done by evaluating different solid phase micro-extraction (SPME) fibres in order to select the one best suited for the extraction of the majority of honeybush tea volatile compounds.
- Optimisation of the chromatographic methods, GC×GC-FID and GC×GC-qMS methods for statistical and qualitative analyses of honeybush tea. This entailed evaluation of different chromatographic columns, oven temperature program, and carrier gas flow rate.
- Determining the difference in volatile composition of three *Cyclopia* species using GC×GC-FID data by principle component analysis (PCA).

ii) Last part of the research was to develop a GC×GC-TOF-MS method for further identification of honeybush tea volatile compounds. The GC×GC system consists of cryogenic dual stage modulator and time-of-flight mass spectrometry (TOF-MS) as a detector.

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1.3. References

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Chapter 2

Literature review

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2.1. Honeybush tea

2.1.1. Introduction

Cyclopia species, belonging to the leguminous family Fabaceae (tribe Podalrieae) are used to prepare South Africa's indigenous tea known as 'honeybush' [1]. *Cyclopia* shrubs are endemic to the Cape fynbos biome and grow in the coastal regions of the Western and Eastern Cape provinces [2]. The name 'honeybush' is derived from the sweet honey-like aroma of the flower of the *Cyclopia* plant. The plants are characterised by their woody stems, deep yellow flowers with indented calyxes and trifoliate leaves which vary from pubescent, narrow to flat shaped, depending on the species [2,3]. Examples of the shrubs of selected *Cyclopia* species are shown in **Figure 2.1**. The use of honeybush as a tea dates back to the 18th century. *C. genistoides* was the first recorded species used for tea preparation [4], followed by *C. vogelli* (later renamed *C. subternata*) [5], *C. latifolia* and *C. longifolia* [6]. The choice of species used for the preparation of tea depended on the prevalence of species in a particular region; according to Marloth, *C. genistoides* was used in the Cape Peninsula, whereas *C. subternata* was used in the Caledon (Overberg) and George areas [6]. To date, more than 20 *Cyclopia* species have been identified, and while some are widespread, others occur in more localised regions, as shown in **Figure 2.2A** [7].

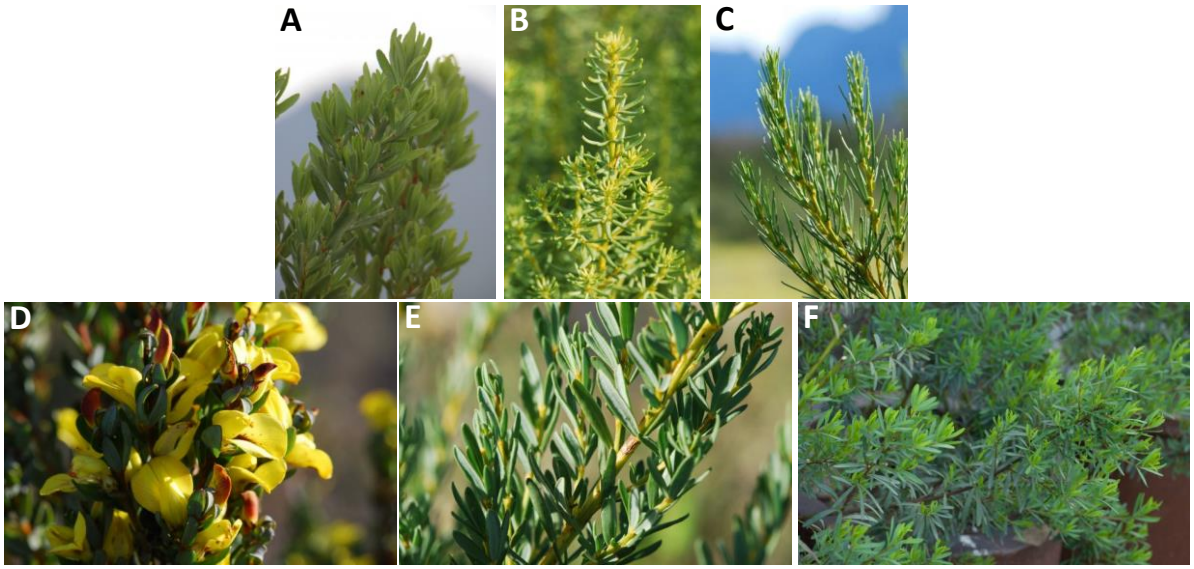

Figure 2.1: *Cyclopia* shrubs showing different leaf shapes for each of the following species: (**A**) *C. intermedia*, (**B**) *C. genistoides*, (**C**) *C. maculata*, (**D**) *C. sessiliflora*, (**E**) *C. subternata* and (**F**) *C. longifolia.* (Adapted from [8]).

Since its natural habitat is prone to fire devastation, *Cyclopia* plants have developed survival strategies which categorise them into either sprouters or re-seeders. Sprouters such as *C. intermedia*, *C. genistoides* and *C. sessiflora* have woody rootstocks (lignotuders) from which the

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new shoots develop after a fire. Re-seeders, for example *C. subternata* and *C. maculata*, lack lignotubers and their survival depends on the scarification of the hard seed coat by fire for the seeds to sprout [3]. Currently, mainly three species, *C. genistoides*, *C. subternata* and *C. intermedia,* are exploited commercially. The industry has grown substantially during the last few years and this, together with frequent veld fires, limits the availability of the plant material and has provided the incentive for the cultivation of additional species [7].

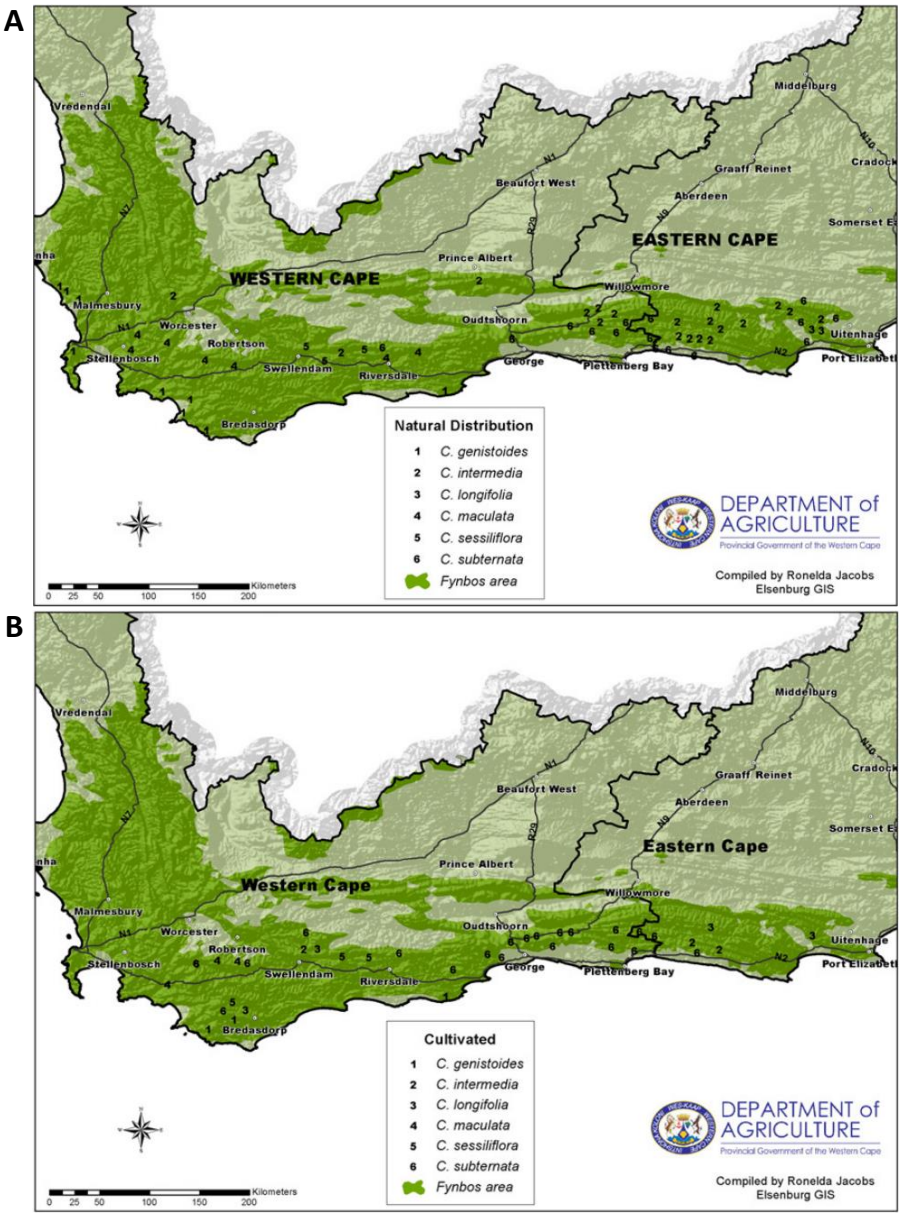

Figure 2.2: Map of the Western and Eastern Cape regions of South Africa showing the (**A**) natural and (**B**) cultivated distributions of the economically important *Cyclopia* species. (Adapted from [7]).

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Figure 2.2 shows the natural distribution and cultivation areas of the most important species. *C. subternata* grows mainly in sandy loam soil and is cultivated in the Langkloof and Riversdale areas and in-land of the Overberg. *C. genistoides* grows naturally in the coastal areas of the West Coast and Mossel Bay and is therefore also cultivated in these areas. *C. intermedia* makes up most of the production of honeybush, however cultivation is not favoured as it can only be harvested every second to third year [7]. Therefore, this species is still predominantly harvested from the wild. The growing demand of honeybush tea worldwide has raised interest in including other species such as *C. sessiliflora*, *C. maculata* and *C. longiflora* for cultivation and tea production.

The popularity of honeybush tea not only stems from its characteristic pleasant, honey-like aroma and flavour but also from its health promoting properties. Honeybush has traditionally been used as a medicine for chronic catarrh [9] and to alleviate stomach ailments such as heartburn and nausea [6]. Studies have also shown that honeybush has antioxidant, anti-mutagenic, anti-cancer and phytoestrogen properties, which are mainly associated with the presence of phenolic compounds in honeybush extracts [10]. Recently anti-diabetic [11] and anti-obesity [12] properties have also been described. In addition, honeybush is a caffeine-free tea, making it desirable amongst health conscious individuals [4].

Honeybush is mainly used as a herbal tea, although a small percentage of the produce is used in food, cosmetics and toiletries. Most products on the market comprise mixtures of two or more *Cyclopia* species or blends of honeybush and rooibos (*Asphalatus linearis*) and/or other indigenous South African plants such as marula (*Sclerocarya birrea*) fruits [10]. Although honeybush has a long history of regional use, the industry has grown substantially since its re-discovery, especially in overseas markets. Currently honeybush is exported to countries such as Germany, the Netherlands, the United Kingdom, Poland and the United States of America (USA) [2,7].

2.1.2. Honeybush tea production

The leaves and twigs of *Cyclopia* shrubs are processed to provide either fermented or unfermented honeybush tea. Processes that follow after harvesting involve shredding of shoots, wetting, fermentation in case of fermented tea, drying and sieving [10]. All these processes have an effect on the quality of the tea produced.

Harvesting is preferably done in summer to late autumn, before flowering occurs, as this puts the plants under unnecessary stress and the flowers do not contribute significantly to the flavour and aroma [2,13]. After harvesting, the plant material is cut to provide fine pieces of 2 to 3 mm in length. This also disrupts the cellular structure to facilitate fermentation. The cut plant material is then pre-wetted with cold water - this speeds up the development of the dark-brown colour during fermentation and also decreases the presence of uncoloured bits of stems in the final product [14].

Traditionally the *Cyclopia* plant material was fermented in heaps [6] or by baked-oven [15] fermentation followed by sun-drying. Heap fermentation, though inexpensive, does not allow effective control of processing conditions and prolonged heap fermentation often yields tea of poor quality due to mould growth and incomplete fermentation [2]. With the demand for high

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quality tea and export regulations requiring low levels of microbial contaminants, the use of heap fermentation was abandoned and alternative methods had to be found. Du Toit et al. have shown that fermentation at higher temperature (>60°C) and controlled drying conditions leads to the production of tea of consistent high quality and acceptable microbial content [13]. Fermentation conditions currently used in the industry include temperatures greater than 60°C and fermentation periods ranging from 18-60 hours, depending on the temperature and species. The plant material is dried directly after fermentation, either in rotary driers under controlled conditions or in the sun on drying racks. The dried material is then sieved into different sizes ranging from a coarse cut to dust (<40 mesh) [2,10].
In the production of green, unfermented honeybush, oxidation and browning of the plant material is prevented *via* a steam-treatment process that inhibits enzyme activity. After the cut plant material is passed through this process, vacuum-drying is used to produce the desired greener product [7].

2.1.3. Chemical composition

Tea quality is essential for market value and it is defined by the sensory characteristics colour, aroma and taste. These properties depend on the chemical composition of the tea; non-volatile compounds like phenolics and sugars are mainly responsible for the colour and taste, while the volatile compounds contribute to the odour and aroma.

Tea, like most natural products, typically contains hundreds of volatile compounds. However, not all of these compounds contribute to the perceived aroma. Volatile compounds are found in food in widely varying concentrations and each has a different odour activity, which can be described by three properties: absolute threshold, intensity as a function of concentration (psychometric function) and quality. The absolute threshold is the minimum concentration above which a given compound can be perceived, and is different for each compound and matrix-dependent. Thus only compounds above their absolute threshold concentrations will contribute to the aroma. **Figure 2.3** illustrates the perceived odour dependency of two compounds on their concentration. Each compound has a unique psychometric function, or concentration-response curve. Generally the intensity of the perceived aroma increases with concentration, until a point where an intensity increase can no longer be perceived (the saturation zone) [16,17]. With regard to the odour quality, some compounds do not have a distinct odour at their absolute threshold, but the quality of the odour increases with concentration.

Individual odour activities of each compound do not predict the perceived overall aroma, since in a complex mixture the contribution of each compound may remain distinct, be suppressed or synergistically produce a different aroma in combination with other compound(s). Furthermore, even compounds below their thresholds or odourless compounds can contribute to the aroma of a complex mix. Clearly, the study of the relationship between chemical composition and aroma is a complex and challenging task.

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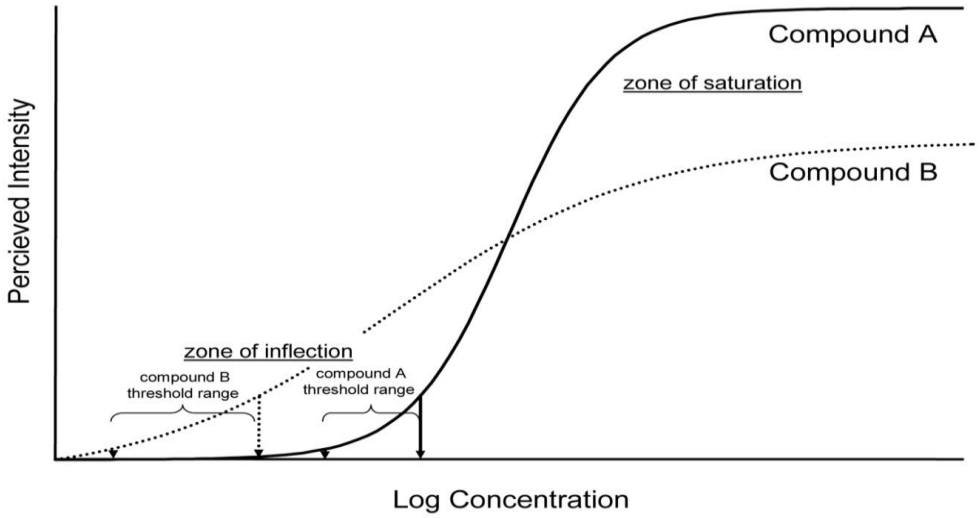

Figure 2.3: Correlation of the concentration and the perceived odour intensity of two different compounds (Adapted from [17]).

Sensory analysis can give a description of aroma attributes, but cannot directly correlate them to specific chemical compounds. In contrast, analytical techniques such as gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) can provide information on the composition of individual volatile compounds. For this reason, chemical and sensory data are often used in combination in order to find possible correlations. Furthermore, coupling GC to olfactometric detection (GC-O) , where a sniff port is used for human detection of odour-active compounds, can provide insights into both the composition and sensory properties of individual compounds [17].

Recently, GC-MS and GC-O were used for the comprehensive chemical characterisation of selected honeybush species. A total of 255 volatile compounds were identified and 46 of these were found to be odour-active by means GC-O. The volatile compounds comprised several different classes of compounds, summarised in **Table 2.1**. Terpenoids were the predominant group, followed by aldehydes, esters and ketones [18].

The same study compared the volatile profiles of 7 honeybush samples of four *Cyclopia* species (*C. intermedia*, *C. genistoides*, *C. subternata* and *C. longilfolia*) quantitatively. *C. subternata* and *C. genistoides* samples originating from different geographical sources were included in the study. It was found that the same volatile compounds were present in all four species, although they differed in their relative concentrations (**Table 2.2**). Differences in volatile composition were observed between all the samples, indicating that not only species, but also region, plays a role in the aroma of the end product. This study contributed significant insight into the volatile composition of honeybush and implies that the volatile content can potentially help distinguish honeybush species and to determine the origin of the species. However no definite conclusions could be drawn regarding these aspects, as a limited number of samples were analysed.

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Table 2.1: Classes of compounds identified in honeybush species by GC-MS (Reproduced from [18]).

Compounds class	Total number of compounds
Aldehydes (saturated, unsaturated, aromatic)	25
Esters (saturated, unsaturated, aromatic)	20
Hydrocarbons (saturated, unstaurated, aromatic)	16
Ketones (saturated, unsaturated, aromatic)	16
Alcohols (saturated, unsaturated, aromatic)	10
Furans (saturated, unsaturated, aromatic)	8
Lactones (saturated, unsaturated, aromatic)	8
Carboxylic acids (saturated, unsaturated, aromatic)	6
Other compounds	4
Ethers	3
Phenols	1
Terpenes	33
Terpene alcohols	30
Terpene aldehydes	9
Terpene ketones	31
Terpene esters	7
Terpene ether	27
Terpene lactone	1

In another study, GC-MS and GC-O were used for the characterisation of *C. subternata* volatiles and to determine the odour active compounds of these species [18,19]. 183 compounds were identified and of these, 37 were odour active (**Table 2.3**). Some of the major odour active compounds found in *C. subternata* were (*E*)-β-damascenone, (*R*/*S*)-linalool, (*E*)-β-damascone, geraniol, (*E*)-β-ionone, and (7*E*)-megastigma-5,7,9-trien-4-one. (*E*)-β-damascone and (7*E*)-megastigma-5,7,9-trien-4-one are described as contributing tea-like and spicy aromas with undertones of dried fruit, while the other four compounds are associated with sweet aroma. The odours of (6*E*,8Z)-megastigma-4,6,8-trien-3-one, (6*E*,8*E*)-megastigma-4,6,8-trien-3-one, (7*E*)-megastigma-5,7,9-trien-4-one, 10-*epi*-*γ*-eudesmol, *epi*-*α*-muurolol and *epi*-*α*-cadinol were perceived as typically honeybush-like (**Table 2.3**).

Another variable that was investigated by several scientists is the role of fermentation (oxidation) on the volatile composition of honeybush. As expected, significant differences have been observed between fermented and unfermented teas. The effect of fermentation on the volatile composition of *C. genistoides* was first investigated by Le Roux et al. [18,20] and the results are summarised in **Figure 2.4**. In this work 77 and 79 aroma compounds were identified in unfermented and fermented samples, respectively. Fermentation did not lead to the production of any new compounds, but affected the relative concentrations of volatiles already present in the unfermented (green) tea. The same was found for *C. intermedia* in a study where a much larger number of volatiles were included [18] (**Figure 2.5**).

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Unfermented *C. genistoides* mainly contained saturated and unsaturated alcohols, aldehydes and methyl ketones, which are characterised by grassy aroma descriptors. On the other hand, in fermented *C. genistoides* samples the most important group was the terpenoids, which are known to possess sweet and floral notes (**Figure 2.4**). The relative concentrations of the main 'green' odour compounds decreased substantially after fermentation, whereas the opposite occurred for the 'sweet' compounds like linalool. Unlike *C. genistoides*, linalool and geraniol were the main aroma-active constituents of unfermented *C. intermedia*. Similar to what was observed for *C. genistoides*, however, their relative concentrations also increased after fermentation (**Figure 2.5**).

Table 2.2: Comparison of relative abundances (% area of TIC chromatogram) of the most intense odour active volatile compounds of four *Cyclopia* species [Adapted from 18]

		C. genistoides		***C. intermedia***	***C. longifolia***	***C. subternata***	
Compound	**Aroma descriptors**	**Albertina**	**Pearly beach**			**Bredasdorp**	**Genadendal**
Linalool	Refreshing, light, clean, floral	29.38	**31.7**	28.88	19.67	23.95	17.41
(*E,Z*)-2,6-Nonadienal	Green-vegetable, cucumber, violet-leaf	0.07	0.11	0.12	**0.22**	**0.22**	0.17
(*E*)-2-Nonenal	Green, cucumber, aldehydic, fatty	0.05	0.07	0.11	**0.12**	**0.13**	0.09
Geraniol	Sweet, floral, rose	12.43	22.5	13.9	**27.61**	25.34	5.1
Component C178	Not available	0.37	0.08	**0.42**	0.09	0.06	**0.42**
(*E*)-β-Damascenone	Woody, sweet, fruity, earthy, green-floral	0.67	**1.37**	1.04	0.72	0.61	0.5
(*E*)-β-Damascone	Fruity (apple-citrus), tea-like, minty notes	0.24	0.4	**0.74**	0.48	0.25	0.45
2,3-Dehydro-γ-ionone	Not available	0.04	0.2	0.09	**0.3**	0.25	0.11
3,4-Dehydro-β-ionone	Ionone-damascone, saffron-like, fruity, leathery	0.16	0.04	0.13	0.12	0.1	**0.46**
(*E*)-β-Ionone	Warm, woody, fruity, raspberry-like	1.43	0.84	1.52	2.5	**3.06**	2.99
10-*epi*-γ-Eudesmol	Woody, floral, sweet	0.06	0.02	**0.59**	0.1	0.12	0.22
epi-α-Cadinol	Herbaceous, woody	0.01	**0.078**	0.063	0.061	0.061	0.064
epi-α-Muurolol	Herbaceous, slightly spicy	0.007	**0.045**	0.043	0.029	0.034	0.034
(7*E*)-Megastigma-5,7,9-trien-4-one	Tea-like, spicy, dried fruit	0.0011	0.0017	<0.001	<0.001	**0.0018**	0.0014

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Table 2.3: Major odour active compound of *C. subternata* identified by GC-O (Adapted from [19]).

Compound	Aroma Descriptors	% Area[a]	DF[b] (%)
(*E*)-β-Damascenone	Woody, sweet, fruity, earthy green-floral	0.607	100
Linalool	Refreshing, floral-woody	23.954	100
***(E,Z)*-2,6-Nonadienal**	Green-vegetable, cucumber, violet leaf	0.223	100
(*E*)-2-Nonenal	Green, cucumber, aldehydic, fatty	0.128	100
(*E*)-β-damascone	Fruity (apple-citrus), tea-like, minty	0.607	93
Geraniol	Sweet, floral, rose, citrus-like	25.344	93
(*E*)-β-Ionone	Warm, woody, fruity, raspberry-like	3.061	87
Component C178	Not available	0.061	60
3,4-Dehydro-β-ionone	Ionone-damascone, saffron-like, fruity, leathery	0.104	87
2,3-Dehydro-γ-ionone	Not available	0.247	87
(7*E*)-Megastigma-5,7,9-trien-4-one	Tea-like, spicy and resembling dried fruit	0.002	60
epi-α-Cadinol	Herbaceous, woody	0.061	60
epi-α-Muurolol	Herbaceous, slightly spicy	0.034	60
10-*epi*-γ-Eudesmol	Woody, floral, sweet	0.117	40
(*E*,*E*)-2,4-Decadienal	Fried, waxy, fatty, orange-like	0.035	33

Most intense odour active compound contributing to honeybush tea aroma are highlighted in bold; [a] % area for TIC chromatogram, [b] Detection frequency.

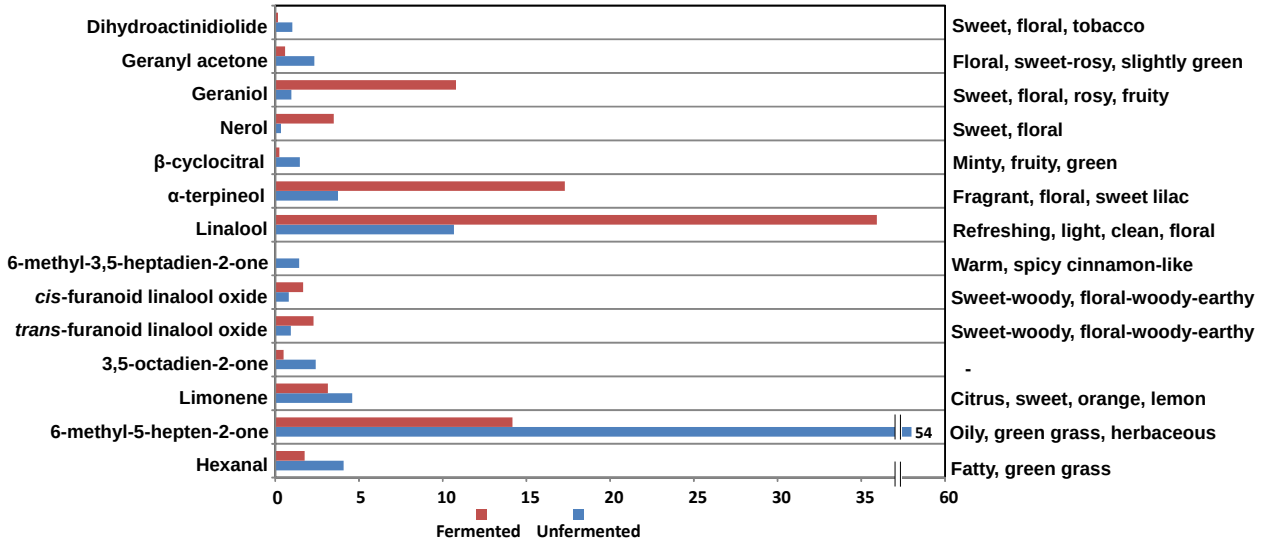

Figure 2.4: Grapical representation of relative area percentage of the main volatile components of unfermentated and fermentated *C. genistoides* with their odour description (Reproduced from [20])

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From the above brief overview, it is clear that important progress has been made in the investigation of the volatile content and aroma of honeybush tea. At the same time, however, much work remains to be done. Although a large number of compounds have already been identified, further work on the chemical characterisation of honeybush volatiles will likely allow identification of new volatile compounds. Furthermore, the analysis of more samples of each species is required to investigate inter-species differences in volatile composition in a more rigorous way, preferably in combination with suitable statistical methods. Finally, more detailed sensory data recently reported for honeybush [21] provides the ideal information to study in detail the compounds responsible for the important aroma properties of this important herbal tea.

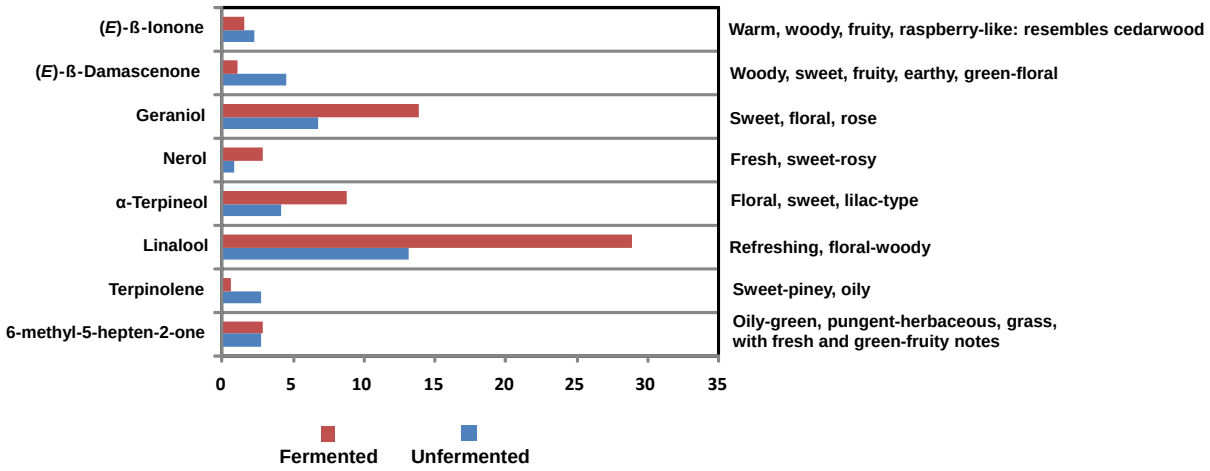

Figure 2.5: Representation of the abundance of the most intense odour active volatile compounds indentified in unfermented and fermented *C. intermedia* (Reproduced from [18])

2.2. Instrumentation

2.2.1. Gas chromatography

Chromatography is a term used to describe a group of separation techniques in which components of a mixture are separated based on their differential distribution between two immiscible phases, i.e. the mobile phase and stationary phase [22].

Gas chromatography (GC) has become an indispensable technique for the analysis of volatiles and semi-volatiles in a large array of matrices. Examples of some of the numerous applications of GC include the analysis of contaminants such as pesticides, pollutants in air, water and soil, plasticisers such as phthalates, petroleum samples, etc. One important application field of GC is in the food and beverage industry for the analysis of volatile constituents responsible for aroma attributes. GC is widely used for quality control and detection of adulteration and, in combination with sensory studies, to investigate the volatile constituents responsible for specific attributes relating to a certain product.

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While the technique has undergone significant developments over the years, the basic principles and instrumental components (**Figure 2.6**) remain the same: analytes present in a sample are volatilised in an injector and carried through the column by a carrier gas, where the compounds are separated by differences in their partial vapour pressures (the apparent vapour pressure at a given temperature and pressure) as well as a number of specific interactions with the stationary phase. As the compounds elute from the column, detection is achieved using any of a range of detectors available for identification and/or quantification of separated compounds. The most important components of a modern GC will be discussed briefly below.

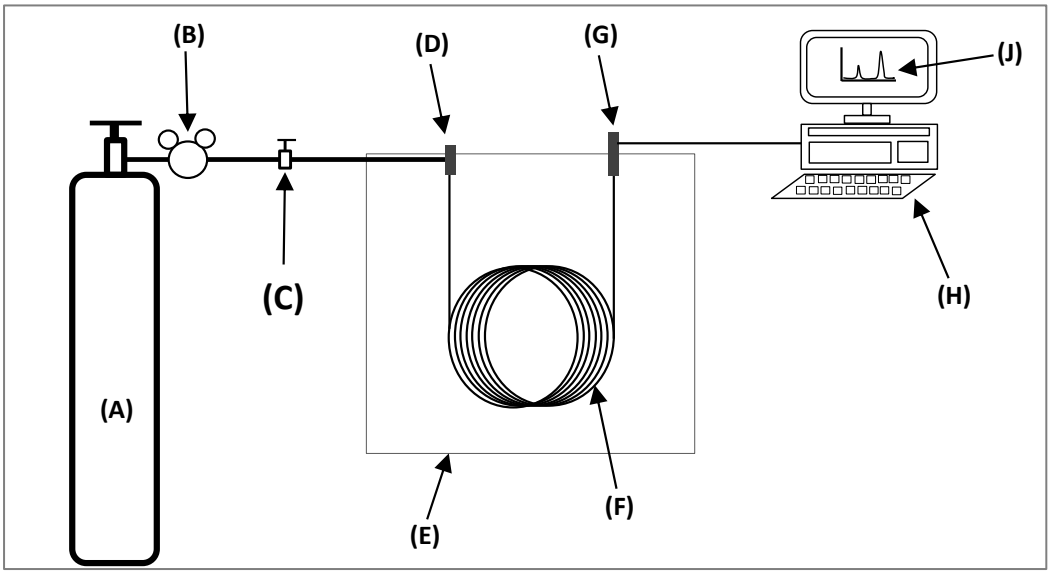

Figure 2.6: Schematic diagram of a gas chromatography instrument. (**A**) carrier gas supply, (**B**) gas cylinder regulator, (**C**) gas flow controller, (**D**) injector, (**E**) oven, (**F**) column, (**G**) detector, (**H**) computer for recording of data and further processing and (**J**) chromatogram representing the results.

2.2.1.1. Injectors

The function of the GC injector is primarily to vaporize the liquid sample and introduce it into the column. It should be noted, however, that samples can be injected in the gas phase as well, or, in the case of solid phase micro-extraction (SPME), analytes are desorbed from the fibre in the hot injector and transferred to the column. The split/splitless injector (**Figure 2.7**) is the most widely used GC inlet. This injector can either be operated in split or splitless modes, as the name suggests. In split mode, only a small portion of the sample vapour is carried by the gas to the column entrance, while the rest is discarded through the split outlet. Split ratios of between 1:10 and 1:100 are most commonly used. This mode is particularly useful to avoid overloading of GC columns and saturation of detectors by highly abundant analytes – in fact, the split/splitless injector was invented for use with low-volume capillary GC columns for this reason. The splitless mode is mainly used for trace analysis; in this mode the split vent is closed for a short period of time to allow all the sample vapour to be carried to the column, followed by opening of the split

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vent to flush the injector (*cf*. **Figure 2.7**). Much higher sensitivity than split injection is thus achieved, although sample focusing mechanisms are often required to avoid injection band broadening during split injection [23].

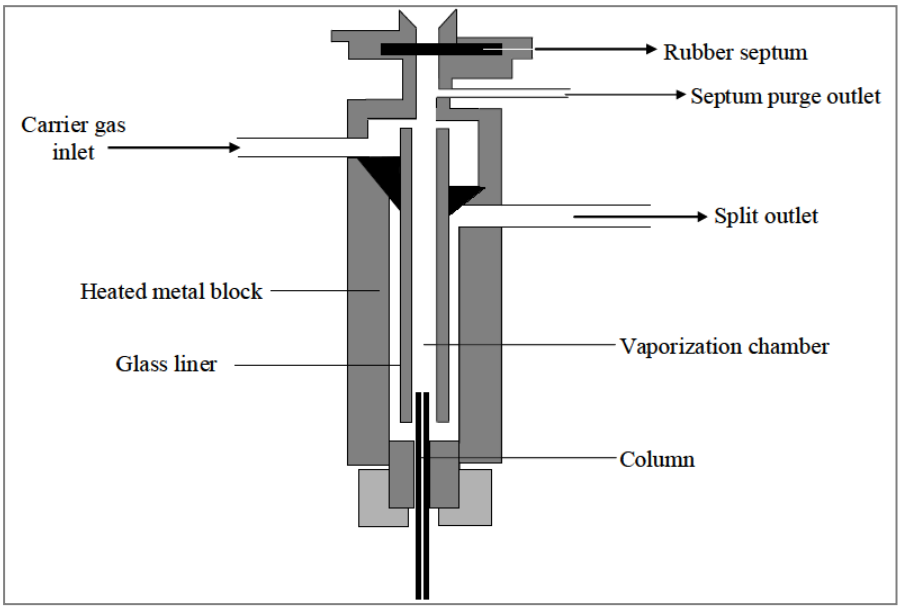

Figure 2.7: Schematic drawing of a spilt/splitless injector (Reproduced from [25]).

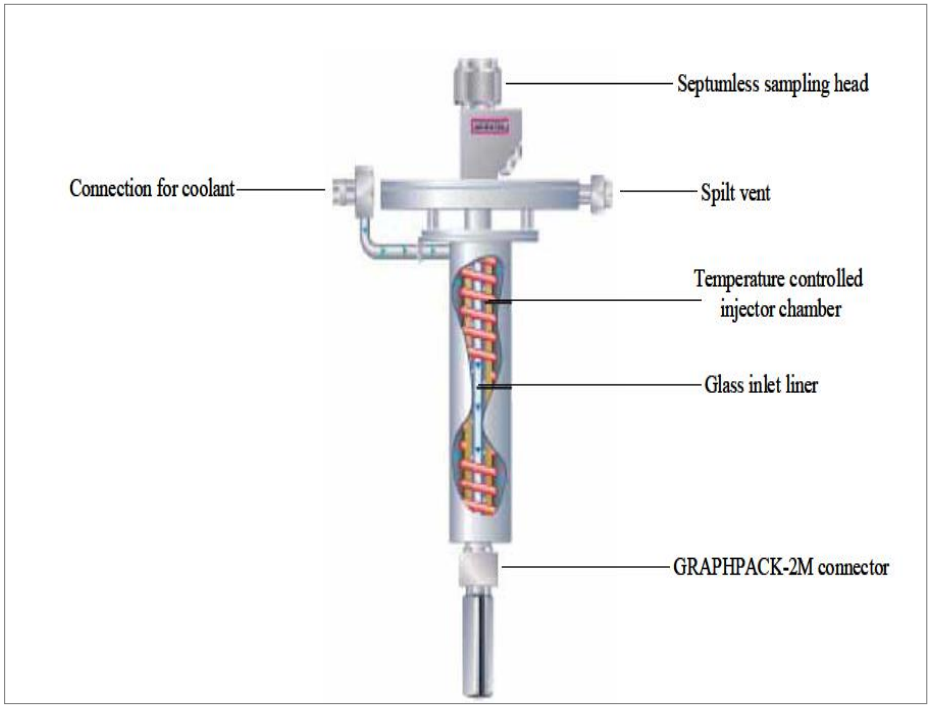

Figure 2.8: Picture of a programmed temperature vaporisation (PTV) injector (Reproduced from [26]).

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A programmed temperature vaporization (PTV) injector (**Figure 2.8**) is similar to a split/splitless injector. In contrast to split/splitless, where a constant temperature is used throughout, the PTV can be temperature programmed. This enables the PTV to be used as a cold 'trap' during slow sample introduction, for instance while desorbing a stir bar when performing stir bar sorptive extraction (SBSE) or when doing large volume liquid injections [23]. Following injection, the injector is heated very rapidly (up to 12^{o}C/sec) for rapid transfer to the column. The PTV offers the advantage of minimising analyte discrimination and also improves the analysis of thermally labile analytes [24].

2.2.1.2. Carrier gas and GC column

In gas chromatography, volatile analytes are transported by an inert gaseous mobile phase through a column which contains the stationary phase. The gas should be inert and not interact with analytes, therefore helium and hydrogen, or in special cases nitrogen, are commonly used. Previously, the choice of the gas depended on the detector being used, but nowadays the cost, speed and the application determines the choice of the carrier gas [22]. The dynamic viscosity of the carrier gas is independent of the pressure, however it does vary with temperature - the viscosity of gas increases with temperature [22]. Thus it is essential to measure and accurately control the gas flow for optimal column efficiency. For these reasons, electronic pneumatic control (EPC) was developed, which offers the advantage of allowing analysis in either constant flow or constant pressure mode [22].

The nature of the stationary phase determines the type of interactions that govern the separation. When the stationary phase is a solid, adsorption is dominant, and when it is a liquid, partitioning occurs. Hence, GC can roughly be divided into two types of separations: gas-solid chromatography (GSC) and gas-liquid chromatography (GLC) [27,28]. GLC, where capillary fused silica columns wall-coated on the inside with a thin polymeric film are used, is by far the most utilised form of GC nowadays. However, GSC still finds application in, for instance, the analysis of very volatile compounds and permanent gases. In the case of capillary GLC, the analytes partition between the stationary phase film and the gaseous mobile phase, and differential partitioning of components depends on the temperature and their physiochemical properties. Components which have high affinity for the stationary phase will spend less time in the mobile and thus elute later [22,28]. Numerous stationary phases have been developed varying in selectivity and polarity. The choice the stationary phase depends mainly on the type of analytes and the matrix of the sample. On a non-polar phase analytes are separated according to their boiling points and based on their polarity on a polar phase. The most extensively used stationary phases are based on the non-polar polydimethylsiloxane (PDMS) and on the more polar polyethylene glycol (PEG or Wax). Generally, PDMS has a low selectivity for polar compounds. In order to accommodate more polar compounds, columns with intermediate polarity PDMS phases are modified by replacing the methyl group with a phenyl group in ratios of 5%, 35% or 50% of phenyl substitution. The more phenyl present the more polar the phase becomes. There are other phases with specific selectivity, such as FFAP (free fatty acid phase), and cyanopropyl phase which are used for the analysis of acidic compounds and compounds of medium polarity,

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respectively. Chiral separation columns composed of cyclodextrins and polysiloxane phases have also been developed for separation of stereo-isomers [28].

Column dimensions namely length (L), internal diameter (i.d.) and stationary phase thickness (d_f), have a great effect on the separation of components. The length of the column affects the retention time and resolution of the analytes. Longer columns give better separation but substantially increases the analysis time. Thicker stationary phase increases the retention of components and minimises overloading of column, and also give better separation, however, longer residence times of the analytes in the column will also increase analysis time. Furthermore, narrower column id's and thinner stationary phase coatings provide better separation efficiency but also lower the loading capacity of the column. [27].

2.2.1.3. Detectors

The function of the detector is to detect separated analytes as they elute from the GC column and generate an electric signal which is converted to readable information. Depending on the operational mechanism, the GC detector either measure the mass of an analyte or its concentration. The intensity of the electric signal is proportional to the amount of analyte. Below the operation principle of detectors used in this study will be discussed: FID, qMS and TOF-MS.

2.2.1.3.1. Flame ionization detector (FID)

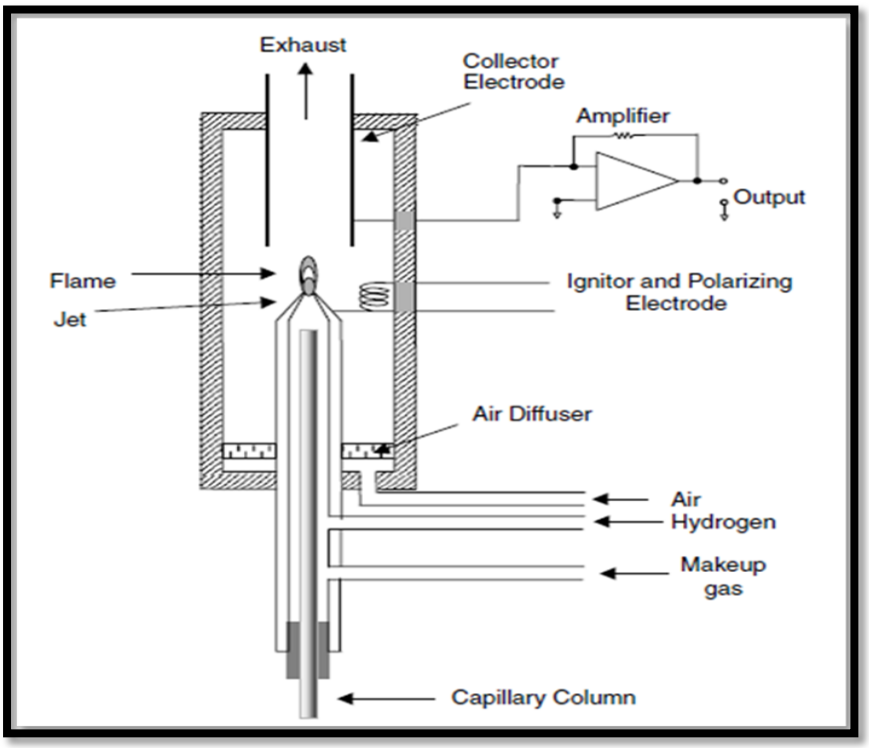

Figure 2.9: Schematic diagram of a flame ionisation detector (Reproduced from [27]).

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The FID is regarded as a universal detector as it can detect almost all the organic compounds. In the FID analytes eluting from the chromatographic column are ionised in a hydrogen flame emanating from the tip of the FID jet (*cf*, **Figure 2.9**). The resulting ions are collected at a collector anode and translated to an electric current which is proportional to the amount of carbon present in the combusted molecules. The FID can only detect compounds containing carbon-carbon or hydrogen-carbon bonds. The FID response depends on the mass of the analyte present, not its concentration, therefore is independent of the gas flow. Organic compounds containing heteroatoms like O, S and halogens reduce the response of the FID, and the detector does not provide a response for permanent gases. The FID is the most widely used detector in GC, largely since it is universal, cheap, relatively sensitive and robust while displaying a wide linear range (~10^7) [27,28].

2.2.1.3.2. Mass spectrometry

Mass spectrometry is extensively used for the identification of compounds separated by GC. Mass spectrometry involves three steps: ionisation, ion separation and detection. Hence a typical MS instrument consists of an ion source to ionise the analytes, a mass analyser to separate charged ions according to their mass-to-charge ratio (m/z) and a detector for detection of separated ions. A range of different ionisation techniques, mass analysers and detectors are available; only the most common and those used in the current study will be briefly outlined below.

The two ionisation techniques that are mostly utilised in GC are electron ionisation (EI) and chemical ionization (CI). The type of ionisation and the relative energies of the produced ions determine the degree of their fragmentation. EI is regarded as hard ionisation technique: in EI, the molecular ions undergo extensive fragmentation and in some cases the molecular ion is not detected. In EI, the molecules eluting from the GC column are bombarded with an electron beam from a heated filament, which ionises the molecules by removing an electron. The electron beam has a standard energy of 70 eV to enable comparison of spectra between instruments. Owing to the high energy of the formed ions, and the fact that EI is performed under low pressure conditions, the resultant molecular ion subsequently undergoes extensive fragmentation. In contrast, chemical ionisation produces less fragmentation than EI, and is known as a soft ionisation technique. In CI, reagent gases such as methane, ammonia or isobutane are used to indirectly ionise the molecules. In essence, the electron beam from the filament ionises the reagent gas, which subsequently collides and reacts with the molecule to form ions. The resulting spectra typically contain the protonated molecular ion (M+1) in high abundance with less fragmentation peaks. After ionisation the charged ions are guided through lenses to the mass analyser for separation [27,29].

MS instruments are classified based on the type of mass analyser used. Two of the most common mass analysers, which were also used in this study, are quadrupole (qMS) and time-of-flight (TOF-MS) systems.

A quadrupole mass analyser consists of four parallel metal rods of which two are electrically connected to a radio frequency (RF) voltage and the opposite two to a direct current (DC) voltage. The RF and DC voltages are continuously varied, and the resultant field for each set of voltages provides ions of a specific m/z with a stable trajectory which allows them to pass through the

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quadrupole to the detector. As both RF and DC potentials are gradually increased, ions of increasing *m*/*z* will pass through and be detected. qMS systems can be operated in either full scan or selected ion monitoring (SIM) modes. Full scan mode is used for untargeted analyses and to identify unknown compounds. In this mode, the mass range determines the scan rate, which typically varies between 2-8 mass spectra per second. SIM is used for selective detection of target analytes since only selected ion are monitored, which ultimately increases the sensitivity [29].

Time-of-flight mass analysers (**Figure 2.10**) function on a relatively simple principle. Ions are separated according to their migration time down a field free flight tube. At the inlet of the TOF analyser, a voltage is applied to the ions, imparting them with the same kinetic energy (KE) and accelerating them into the flight tube. Since ions with different m/z values have the same KE, they will travel through the drift region to detector at different velocities. Ions with low m/z values will travel faster than ions with higher m/z values. TOF-MS systems can only be operated in full scan mode due this mode of operation. TOF-MS systems are capable of high resolving power, allowing accurate mass measurements to be performed. The non-scanning nature of TOF-MS systems can also provide high acquisition rates (up to 500 Hz): the scan rate is limited only by ion pulse frequency and spectrum storage speed [27,29]. For high-speed acquisition, however, the high resolution capabilities of the TOF are sacrificed.

An electron multiplier is the most commonly used MS detector, especially on qMS systems. It consists of series of dynodes which are operated at voltages ranging from 1- 3 kV. Separated ions from the analyser collide with these dynodes and are translated to an electric current. TOF-MS systems often use multichannel array for detection instead of electron multipliers [27].

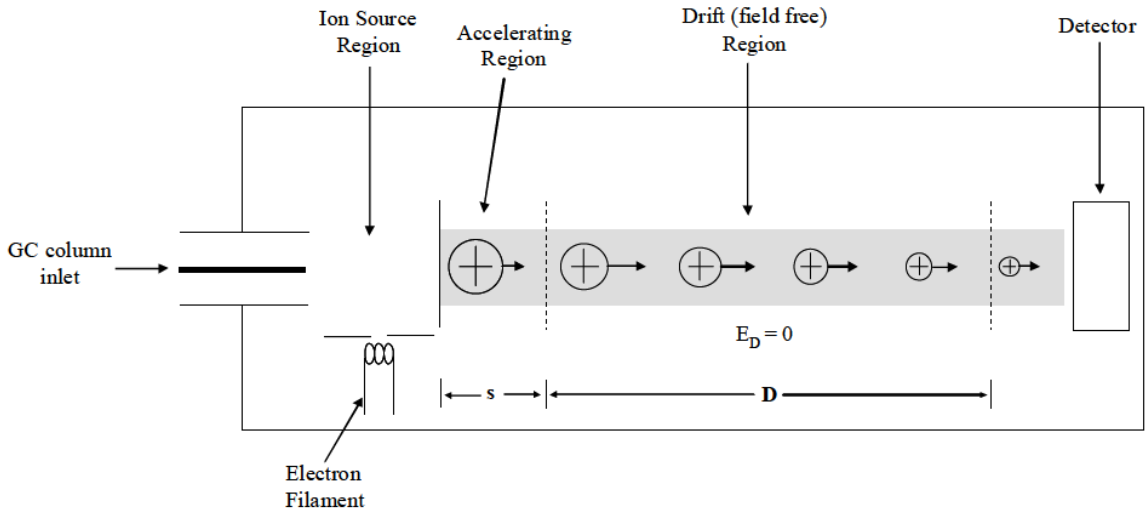

Figure 2.10: Components of a time-of-flight mass spectrometer (Reproduced from [30]).

2.2.2. Multidimensional gas chromatography (MDGC)

The performance of a chromatographic system can be evaluated using the concept of peak capacity. Peak capacity is defined as the number of peaks that can theoretically be separated within the available separation space [31]. Capillary gas chromatography offers very high

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chromatographic efficiency, and therefore high peak capacities compared to most other chromatographic techniques. However, even using highly optimised GC methods, it is often not possible to separate all the components of very complex samples. One way to improve the separation performance is to use multidimensional separation techniques. Here components separated in a given column are subsequently subjected to additional separation(s), ideally based on different retention mechanisms to improve the resolution of co-eluting compounds. Multidimensional gas chromatography (MDGC) can be performed in two different modes: either heart-cutting multidimensional gas chromatography (GC-GC) or comprehensive two-dimensional gas chromatography (GC×GC) [32]. In GC-GC only certain predefined region(s) of the chromatogram obtained in the first column are introduced into the second column. This mode is used to obtain additional information about a particular fraction or fractions of the sample [33]. In GC×GC on the other hand, the entire sample is introduced into the second column for further separation [32]. Both of these modes provide higher peak capacities than one dimensional GC. The peak capacity for a GC-GC separation is the sum of the peak capacities of the two columns, whereas for GC×GC it is the product of the peak capacities of the two columns, as illustrated schematically in **Figure 2.11.**

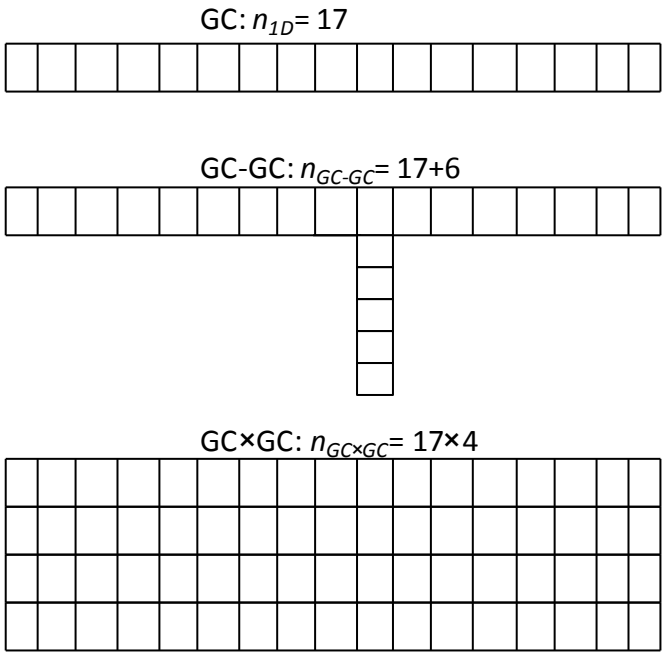

Figure 2.11: Schematic comparison of peak capacities in one-dimensional chromatography (n_{1D}), heart-cutting ($n_{GC\text{-}GC}$) and comprehensive two-dimensional gas chromatography ($n_{GC\times GC}$) (Adapted from [34]).

2.2.2.1. GC×GC instrumentation and operation

GC×GC requires that the entire sample be separated in two columns. The two columns are typically connected in series by an interface called the modulator (*cf*. **Figure 2.12**). The function of the modulator is to sequentially collect effluent from the first dimension column and re-inject it

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into the second dimension column. For the system to function in a truly “comprehensive” manner, it is required that the different separation mechanisms are employed in each column to achieve “orthogonal” separations. In GC×GC this is achieved in practice by using two columns coated with different stationary phases, typically apolar and polar columns. It is also important to ensure that the components separated in the first dimension should remain separated after modulation [35,36]. For the latter to hold true, sufficiently short modulation periods should be used (see **Figure 2.13**).

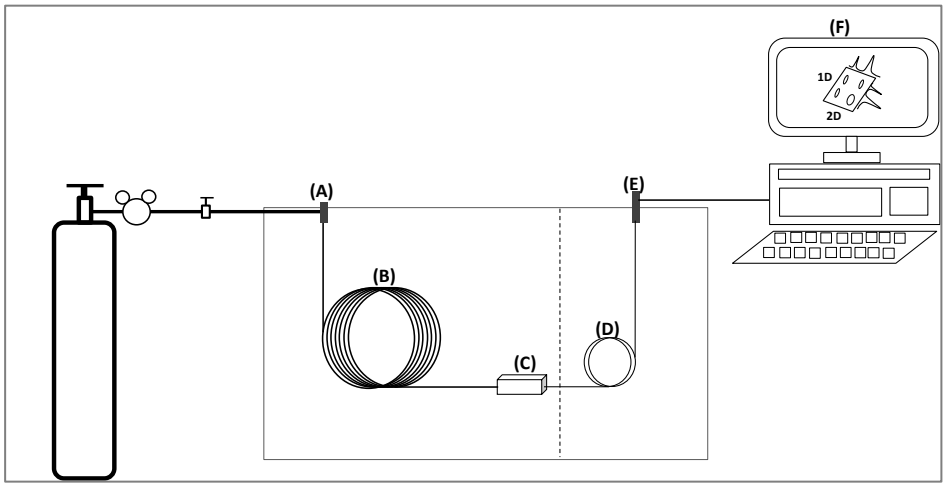

Figure 2.12: Schematic diagram of a GC×GC instrument: (A) injector, (B) first dimension column (housed in the primary column), (C) modulator, (D) second dimension column (can either be installed in the primary oven or in a separate secondary oven), (E) detector and (F) data collecting system.

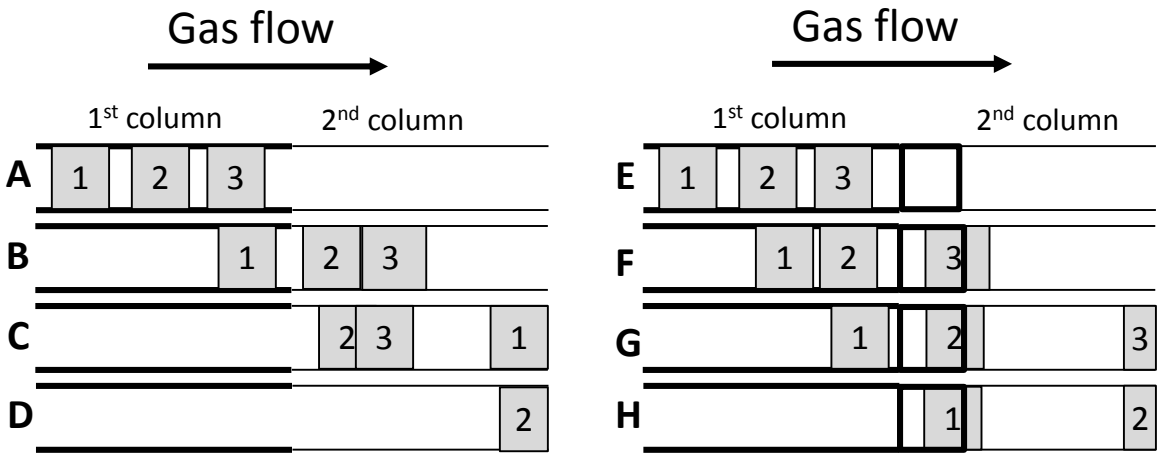

Figure 2.13: Illustration of the hypothetical separation of three compounds on two columns with (left, **A-D**) no modulator, and (right, **E-H**) with a modulator installed between the columns (Adapted from [37]).

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The importance of the modulator may be illustrated graphically as shown in **Figure 2.13** according to Górecki et al. [37]. When two columns with different stationary phases are connected in series without a modulator (**Figure 2.13A-D**), compounds 2 and 3, which were separated in the first column, co-elute after passing through the second column (**Figure 2.13B-D**). Due to the different stationary phase in the second column, compound 1 migrates faster than compounds 2 and 3 in this column (**Figure 2.13C**). In contrast, **Figures 2.13E-H** show the hypothetical situation when the same two columns are connected by a modulator. All three compounds are separated in the first column; compound 3 is trapped first, focused into a band (**Figure 2.13F**) and then re-injected into the second column (**Figure 2.13G**) while compound 2 is being trapped. Only after compound 3 has eluted from the second column, is compound 2 released and injected into the second column resulting in separation of compound 2 and 2' (**Figure 2.13H**). Sequentially the next compound is trapped, focused and re-injected. This mode of operation ensures that separation in both dimensions is preserved. (Note though that in this example the second column provides no additional separation, since all three compounds were already separated in the first column).

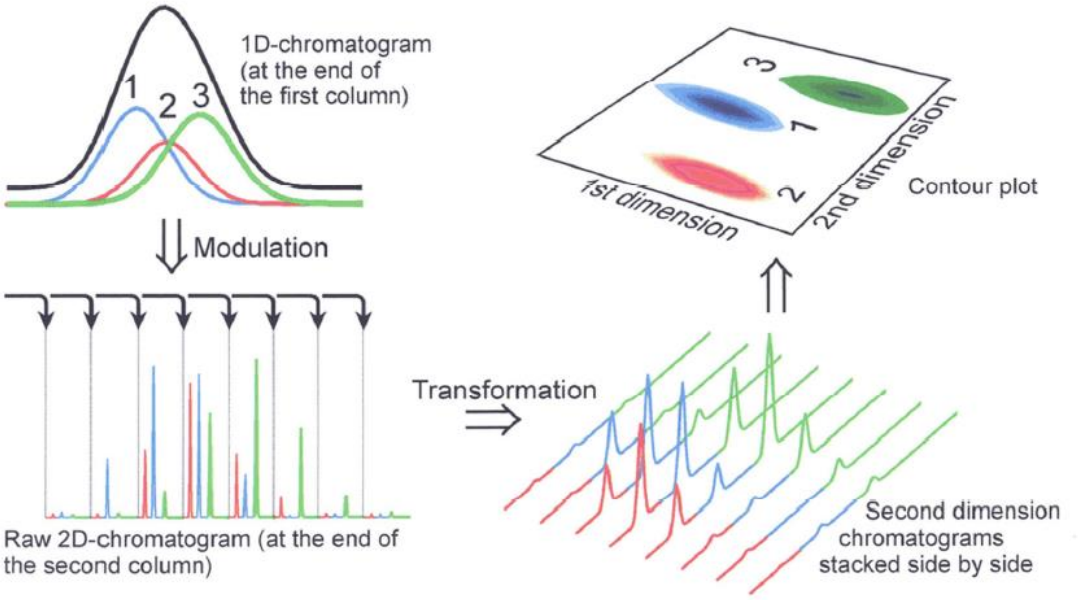

Figure 2.14: Illustration of hypothetical GC×GC separation illustrating how co-eluting compounds 1-3 are modulated and analysed in the second dimension (left). The bottom left figure shows the raw GC×GC data, which is comprised of several consecutive second dimension separations. The figures on the right illustrate how this raw data is manipulated to present the data in a contour plot format. (Reproduced from [36]).

GC×GC requires all of the basic hardware needed for a conventional GC instrument, with the addition of a modulator and an optional secondary oven for the second dimension column. A schematic diagram of a typical GC×GC instrument is shown **Figure 2.12**. Most often a non-polar × polar column configuration is used: a long non-polar column and short polar column in the first

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and second dimensions, respectively. The second dimension separation needs to be very fast to preserve the separation of the first dimension column. According to Murphy et al., a first dimension peak should be sampled at least three times in order to preserve the first dimension separation [38]. Thus modulation periods (i.e. the time taken to complete a single separation cycle in the second dimension) of about 3-8 seconds are commonly employed in GC×GC. If the modulation period is too short, compounds which are highly retained in the second dimension column might elute in the next modulation cycle (this phenomenon is referred to as wraparound) [32]. On the other hand, when the modulation period is too long, compounds which were partially separated in the first dimension column will be re-combined during the modulation step. Therefore, to ensure optimum GC×GC performance thorough optimisation of the several inter-related operational parameters is required. Some of the important optimisation aspects will be discussed in detail in the sections to follow.

The raw data obtained from a GC×GC experiment comprises a series of short consecutive second dimension separations. To visualise the data, these are placed side by side in a matrix format, and the data are typically presented either as a contour plot (**Figure 2.14**) or as a three-dimensional surface plot.

2.2.2.2. Modulation and modulators

A number of modulators have been developed, and these can be divided into two main classes: thermal and flow-based modulators. Thermal modulators work on the basis of rapidly trapping analytes and/or releasing them using differences in temperature. Accordingly, the two sub-classes of thermal modulators are heater-based and cryogenic modulators. In a typical cryogenic modulator, for example, analytes are trapped by decreasing the temperature at the end of the first dimension column or the start of the second dimension column. At the same time the downstream section of the modulator is rapidly heated to release trapped analytes from this section. These are so-called dual stage modulators, since cold and hot spots are sequentially created in different parts in the flowpath. Single-stage modulation, on the other hand, implies that only one section of the flowpath is sequentially heated or cooled [32]. Valve-based modulators utilise multi-port valves to alternately trap and elute fractions of the first dimension effluent. A brief overview of the development of GC×GC modulators will be given in the sections to follow; particular emphasis will be placed on the modulators used in this study.

2.2.2.2.1. Thermal modulators

Heater-based thermal modulators

The very first working modulator was a heater-based system developed by Liu and Phillips [39]. The modulator comprised a segment of a thick-film capillary coated with a conductive paint. Analytes eluting from the first dimension column were trapped in the thick-film stationary phase of the modulator (at the oven temperature), and then desorbed from the capillary and re-injected into the second column by application of an electric pulse to the capillary to heat it. The modulator was then cooled by simply switching off the electric pulse. In this way analytes were continuously sequentially trapped and injected onto the second column. This was therefore a single-stage

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modulator. It did however have some limitations most notably that it suffered from analyte breakthrough: in the period when the trapped analytes are desorbed from the modulator, analytes eluting from the primary column pass through it without been trapped. Subsequently irregular shaped peaks were generated.

To solve this problem a dual-stage modulator was developed [39]. The modulator used the same stationary phase but consisted of two trapping segments. This worked on the basis that when one segment is being cooled to trap analytes, the other is heated to re-mobilise the trapped analytes, and *vice versa*. This successfully prevented analyte breakthrough, although the modulator capillary was not robust and did not deliver reproducible results.

Several other, similar heater-based modulators have been developed. Recently, a dual stage heater-based modulator was developed by Górecki's group [37,40]. This modulator uses a stainless steel (that can be resistively heated) capillary, coated on the inside with a 1 µm layer of polydimethylsiloxane (PDMS). The modulator is mounted outside the GC oven, thereby allowing analytes to be trapped at ambient temperature. Trapping and re-mobilisation of the trapped analytes from the two segments of the capillary (according to the dual stage mechanism outlined above) was achieved by forced air cooling and resistive heating using a capacitive discharge power supply, respectively. This modulator was found to be robust and subsequently led to the development of a single-stage heater-based modulator by the same group [41].

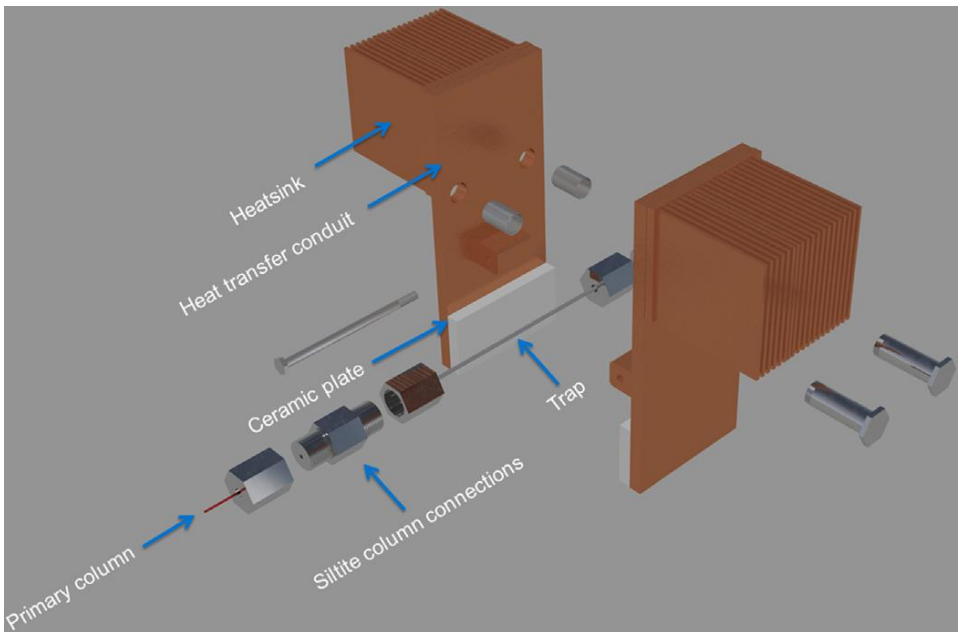

Figure 2.15: A schematic drawing showing the main components of a single-stage thermal modulator developed by Górecki's group (Reproduced from [14]).

To avoid breakthrough from occurring on this single-stage modulator, a section of narrow-bore deactivated fused silica was installed before the modulator capillary to serve as 'restrictor' (*cf*. **Figure 2.15**). This effectively slows down the first dimension carrier gas flow in the restrictor during the heating step used for desorption, thereby preventing analytes from the first dimension

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column from entering the modulator and causing breakthrough [41-43]. The trapping capillary is installed between two ceramic pads connected to heat sinks outside the oven. This keeps the trap at a lower temperature than the oven to facilitate trapping. For re-injection onto the second dimension column the capillary is resistively heated for a short period of time using a capacitive discharge.

Cryogenic modulators

Cryogenic modulators use either solid carbon dioxide (CO_2) or liquid nitrogen (N_2) to cool a portion of the modulator capillary in order to trap the analytes at sub-oven temperatures. The trapped analytes are re-mobilised by either removing the cold spot to a different position or interrupting the delivery of the cooling agent, thereby allowing the oven temperature (or an optional hot-air jet) to facilitate desorption. These modulators offer the advantage of using higher oven temperatures than possible with heater-based modulators, and also trap highly volatile compounds more efficiently [32].

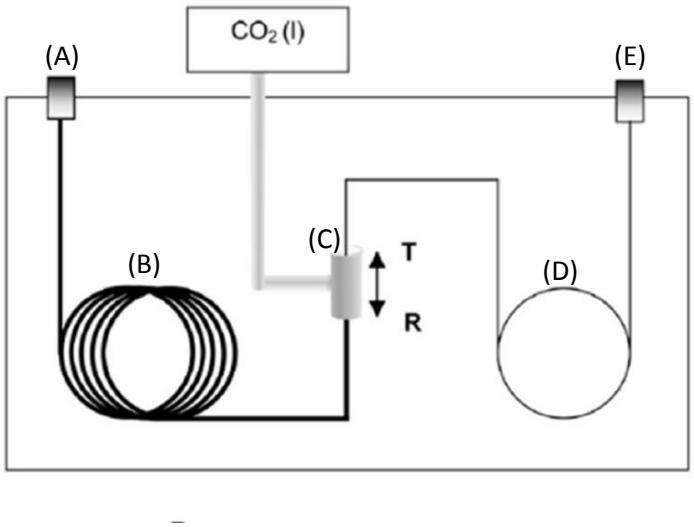

Figure 2.16: Schematic instrumental components of LMCS (Adapted from [9]). (A) Injector, (B) first dimension column, (C) modulator, (D) second dimension column and (E) detector.

Marriot and Kinghorn developed the first cryogenic modulator, called the longitudinally modulated cryogenic system (LMCS) [44]. The modulator contains a movable CO_2 cooled trap, which is moved back and forth between two positions towards the end of the primary column. The analytes are trapped when the cooling trap is placed above their position in the first dimension column. For example, when the trap is moved to the T position (**Figure 2.16)**, analytes in this region are trapped. They are re-mobilised into the second column by moving the CO_2 trap to the R position (i.e. upstream of T), allowing position T to heat up to the oven temperature. At the same time analytes eluting from the first dimension column are trapped in position R, thus preventing breakthrough. The use of moving parts poses several potential disadvantages, such as breaking of the columns and limited lifetime.

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These concerns led to the development of a cryogenic modulator without moving parts by Ledford [45]. This modulator also makes use of CO_2 to trap the analytes, and either hot air jets or oven temperature is used to re-mobilise the analytes. By using two cooling jets, the modulator functions as a dual stage system. This modulator was commercialised through Zoex Corporation, and LECO subsequently modified the system by using liquid N_2 instead of CO_2. **Figure 2.17** demonstrates the operation of the dual jet liquid N_2 modulator, which is widely used currently. Several alternative cryogenic modulators have also been developed, as outlined in papers by Górecki's group [32,37]. In general, cryogenic modulators provide very efficient modulation. A major disadvantage of these systems is however the high operating cost of the instrument, due to the use of costly cryogenics.

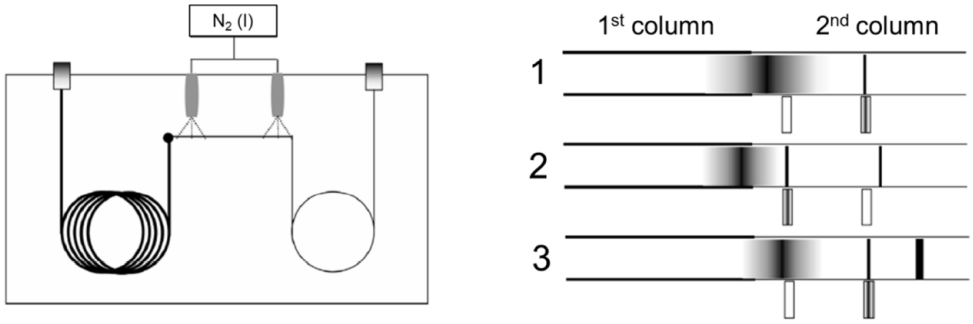

Figure 2.17: Schematic figure demonstrating the configuration (left) and operation principles (right) of a dual stage cryojet modulator. In (1) an analyte band is trapped by the right-hand (second) cooling jet, while the left-hand (first) jet is turned off. In (2) the analyte band is released into the second dimension column by turning off the second jet. At the same time the first jet is turned on to trap the next analytes eluting from the first dimension column. In (3) the first jet is turned off again and the band is passed on to the second jet, which is turned on for sequential trapping. (Adapted from [37] and reproduced from [46], respectively).

2.2.2.2.2. Flow-based modulators

Valve-based modulators

Valve-based modulators make use of multi-port valves equipped with sampling loops to collect and re-inject analytes from the first to the second dimension column. The first valve-based modulator made use of four ports of a two position 6-port diaphragm valve. In sampling mode the analytes from the first dimension column are collected while the previous fraction is separated in the second dimension column. After sampling, the valve switches to the second position, where a small fraction of the effluent is injected into the second dimension column while the rest of the effluent is vented into the atmosphere. During this stage an auxiliary gas is supplied to maintain the gas flow in the second dimension column [47]. Although this configuration served as an alternative to thermal modulators, only a small fraction (10-20%) of the sample was injected into

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the second dimension column, the rest being vented to waste. Furthermore, the operating temperature of the valve limited the use of this modulator for a wide range of applications.

Differential flow based modulators

Much more recently, differential flow modulators have been developed by several groups. These modulators do not incorporate a valve directly in the flow path, but pressure from an external source is used in combination with a two-way valve to stop the flow before and after the modulator. Some systems make use of an additional second sampling loop; the sample is first collected into a loop while the high pressure auxiliary gas is allowed into the second dimension column, during which time the second dimension separation occurs. The flow is then switched to allow the flow of auxiliary gas to flush the analytes into the second dimension column. This modulator allows total transfer of the sample into the second column, and since the valve can be situated outside the oven the modulator could also be operated at higher temperatures [48].

Another type of flow based modulator, referred to as a stop-flow modulator, was introduced by Górecki's group [49,50]. This modulator does not require a loop, but functions by periodically stopping the flow from the first dimension while allowing the separation in the second dimension column to occur. Thermal trapping was used in this modulator. During the stop-flow period, the second dimension column is supplied with an auxiliary gas flow. The flow was pneumatically controlled by solenoid valve placed outside the oven. When the valve was open, the pressure was applied to the outlet of the first dimension column to stop the flow in the first dimension.

Another variant, called the 'pulse flow modulator' [51] similarly functions without the need for a sampling loop. Here a differential pressure pulse exerted by the use of a three-way valve is used to stop the flow from the first dimension before the modulator, allowing the second dimension separation to take place. Thereafter, the flow is stopped after the modulator (before the second dimension column) while the next fraction is being loaded in the modulator. Many other flow modulators have been developed and they are reviewed in [52]

2.2.2.3. Column selection in GC×GC.

GC×GC uses the same types of columns as the conventional capillary GC, although the columns in the first and second dimensions typically differ in terms of their dimensions. Normally a long first dimension and a short second dimension column are employed. The type of stationary phase and the dimensions of the column, i.e. length (L), internal diameter (i.d.) and film thickness (d_f) have a significant effect on the separation performance.

In the first dimension columns with lengths ranging from 15-30 m are typically used. Generally, increasing the column length gives better separation, but also increases the analysis time. The separation in the second dimension has to be very fast – determined by the modulation period, which is typically 3-10 s. Therefore shorter columns with lengths of 0.5-2 m are used in this dimension [53]. First dimension columns of i.d. 0.25 mm are most common, whereas narrow-bore (0.1-0.15 mm) columns are often used in the second dimension to provide better performance for very fast separations [54]. When using a narrow bore column in the second dimension,

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overloading of the column and therefore loss in performance represents a potential concern. Using columns with same phase ratio in both dimensions provides good performance and reduces the risk of overloading of the second dimension column [54]. Generally, thicker stationary phases result in increased retention and relatively broader peaks. This is beneficial in the first dimension of GC×GC to relax the sampling rate requirements and can also help to reduce second dimension column overloading [55].

One of the requirements to ensure successful GC×GC separation is that the columns which are employed should be 'orthogonal': the separation mechanisms dominant in the first and second dimension columns should be different to exploit the two-dimensional space effectively. However, in GC×GC the retention mechanisms are always to some extent related to analyte vapour pressures, and therefore partially correlated [56,57]. Most commonly a non-polar × polar column set is used, where components are separated according to their volatility (or boiling points) in the first dimension and according to polarity in the second dimension. Numerous stationary phases differing in their polarity are readily commercially available. However, there is no universal column set which can be used in all applications – the choice of column set depends on the sample type. For example, in the case of petrochemical samples, the reverse polar × non-polar column combination has proven effective for the separation of aliphatics [58]. 100% polydimethylsiloxane or 5% diphenyl/95% polydimethylsiloxane columns are most commonly used as non-polar phases, and for polar phases 100% polyethyleneglycol or 50% diphenyl/50% polydimethylsiloxane are mostly used. Other columns which offer different selectivity have been used in various applications [53].

2.2.2.4. Detection in GC×GC

As separated components elute from the second dimension column, they must be detected. In GC×GC, the fast second dimension separation and the modulation produce very narrow peaks with peak widths ranging from 50 to 100 ms. In order to accurately measure these peaks, at least ten data points per peak are needed. Therefore a very fast detector with a small internal volume and high acquisition rate (minimum of 50 Hz) is required [59]. The FID is the most commonly used detector for GC×GC as it is capable of acquisition rates as high as 200 Hz. This detector is mostly used for system optimisation, to test performance, or to quantify separated compounds [53]. However, the FID does not provide any structural information, and MS is therefore increasingly being hyphenated to GC×GC separation. Currently, time-of-flight mass spectrometry (TOF-MS) is the only MS system capable of providing sufficiently high acquisition rates (500 Hz) for detection in GC×GC. Although expensive and not readily available to most laboratories, TOF-MS offers many advantages such as spectral deconvolution of partially co-eluting peaks. The latest generation of qMS systems provide higher scan speeds and have therefore also found some application in GC×GC, although to date still much less than TOF-MS systems.

Element-selective detectors such as microelectron capture detector (ECD), nitrogen chemiluminescence detector (NCD), sulfur chemiluminescence detector (SCD), nitrogen phosphorus detector (NPD), atomic emission detector (AED) and miniaturized pulsed discharge detector (MPDD) have also been used in combination with GC×GC [53]. The ability of a detector to rapidly detect peaks depends on the design of the electronics, flow-path and make-up gas introduction, the type of detector response mechanism and the chemistry of the response [60].

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2.3. Sample preparation

For human consumption, a cup of tea is typically prepared by adding boiled water to dried tea leaves and stems. When determining tea quality by sensory analysis, a similar approach is followed in order to recreate the typical sensory profile of the tea as it is consumed. However, for chemical analysis direct injection of the aqueous liquid extract can lead to several instrumental complications and is therefore mostly avoided. For GC in particular, it is not advisable to perform direct injection of the tea extract for number of reasons: (i) the presence of water can cause rapid degradation of the stationary phase of the GC column by hydrolysis and thereby lead to sub-standard performance of the GC system, (ii) the concentrations of odour-active volatile compounds in natural products vary widely but are generally low (typically ppt to ppm levels) [61-63], therefore analytes need to be pre-concentrated before analysis in order to be detected, and (iii) non-volatile compounds present can contaminate the GC injector and the column. For these reasons, sample preparation is essential prior to the GC or GC×GC analysis of tea. Several sample preparation techniques have been described to extract volatile compounds from various matrices. Those that have been extensively used for the analysis of tea and other natural products specifically will be discussed in more detail.

2.3.1. Liquid-liquid extraction (LLE)

In LLE, an organic solvent which is immiscible with water is used to extract volatile analytes from the aqueous sample. During the extraction process, analytes partition between the aqueous and organic layers according to their respective distribution coefficients. LLE, in its most basic form, is simply performed in a separation funnel where the mixture is shaken before the layers are allowed to separate. The organic solvent is removed and often evaporated to a few µL to further concentrate the extract. The latter practice unfortunately may result in the loss of some highly volatile compounds. Solvents such as dichloromethane (DCM), diethyl ether, chloroform, pentane or hexane, or mixtures of solvents are most commonly used. The LLE procedure is relatively simple and has been widely applied. However the main drawback is that is that LLE is not very efficient, especially for more polar compounds. Further disadvantages include the possibility that non-volatile analytes may also be extracted and subsequently contaminate the GC injector and column, the use of large quantities of toxic solvents, formation of emulsions and the labour-intensive nature of manual LLE [27,63,64].

2.3.2. Distillation

Distillation techniques are one of the most commonly extraction methods used in the analysis of plant material. In a traditional direct distillation process, the solid sample is placed in a flask and immersed in water. The sample is then heated directly while stirring to carry over the vapours of distillable components. However, the direct heating of the sample poses the risk of decomposing some sample components. To solve this problem, indirect heating can be employed. In indirect distillation, the steam and the volatile compounds are condensed in a series of traps cooled with a succession of coolants like ice water or dry ice with acetone or methanol. Once the distillate has been collected, the volatiles are extracted by LLE or the extract is further evaporated to concentrate it.

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Nowadays, the most popular and efficient distillation technique for extraction is simultaneous steam distillation/extraction (SDE). SDE offer the advantage of simultaneous isolation and concentration of volatile compounds in a single step. In SDE the apparatus described by Likens and Nickerson [65] is used. In this set-up, steam and an immiscible organic solvent are continuously recycled. The volatile components together with the steam are carried from the aqueous phase to the organic phase. A commonly used solvent is dichloromethane. Generally SDE provide high recoveries, but a potential drawback of this technique that artifacts may be formed due to thermally induced reactions. As a partial solution, SDE can be performed under reduced pressure to discourage thermal decomposition [63,64,66].

2.3.3. Solid phase extraction (SPE)

SPE is an extremely versatile sample preparation technique often used to obtain higher sensitivity not attainable by LLE. Although not commonly used for solid natural product samples, it can be useful to extract compounds of interest from infusions prepared from such samples. SPE makes use of a (commercial) cartridge which is packed with a solid stationary phase. Numerous stationary phases are available; the specific phase used will depend on the chemical nature of the compounds of interest. This selective technique is particularly beneficial in situations where only a certain group of chemically similar characteristics is targeted. A typical SPE procedure involves the following steps: (i) the cartridge is conditioned with an organic solvent, (ii) the sample is loaded onto the cartridge, (iii) the cartridge is washed to get rid of unwanted compounds or contaminants while the compounds of interest are retained, and (iv) the retained compounds are eluted with a suitable solvent. This extract can be either used directly, or, further evaporated for pre-concentration [67].

2.3.4. Sorptive Extraction

Sorptive extraction refers to sampling methods where the extracted compounds are retained within a polymeric phase that occurs at room temperature above its glass transition point. This differs from adsorptive techniques where analytes are temporarily bound to active sites on the surface. Therefore, in the case of sorptive extraction, a partitioning mechanism applies, very similar to the mechanism that governs LLE. The most well-known and extensively used phase for sorptive extraction is polydimethylsiloxane (PDMS). PDMS has been used in many applications such as open tubular traps (OTTs), SPME, stir bar sorptive extraction (SBSE) and the sample enrichment probe (SEP). Of these, SPME is by far the most utilised. One of the main advantages of sorptive extraction of volatiles is that no solvent is required. A disadvantage of using PDMS, however, is that it is non-polar in nature and therefore performs much better for apolar compounds than for polar compounds, especially if the latter are present in trace levels.

2.3.4.1. Solid phase micro-extraction (SPME)

SPME, the most widely used sorptive extraction method today, was developed by Pawliszyn et al. in 1990 [68]. Originally, the SPME fibre, made of fused silica, was coated with a thin polymeric film of PDMS for extraction of organic compounds from aqueous matrices. During sampling compounds are concentrated in the PDMS by partitioning between the fibre coating and the

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sample, ideally under equilibrium conditions. After sampling the fibre is removed from the sample and compounds are thermally desorbed in a hot GC injection port. The fibre is housed in a syringe-type of holder to protect the fibre and to facilitate the retraction or exposure of the fibre during sampling and desorption processes, and also minimize the potential loss of volatile compounds during the process. (*cf.* **Figure 2.18**). SPME can easily be performed manually, although nowadays it is mostly completely automated.

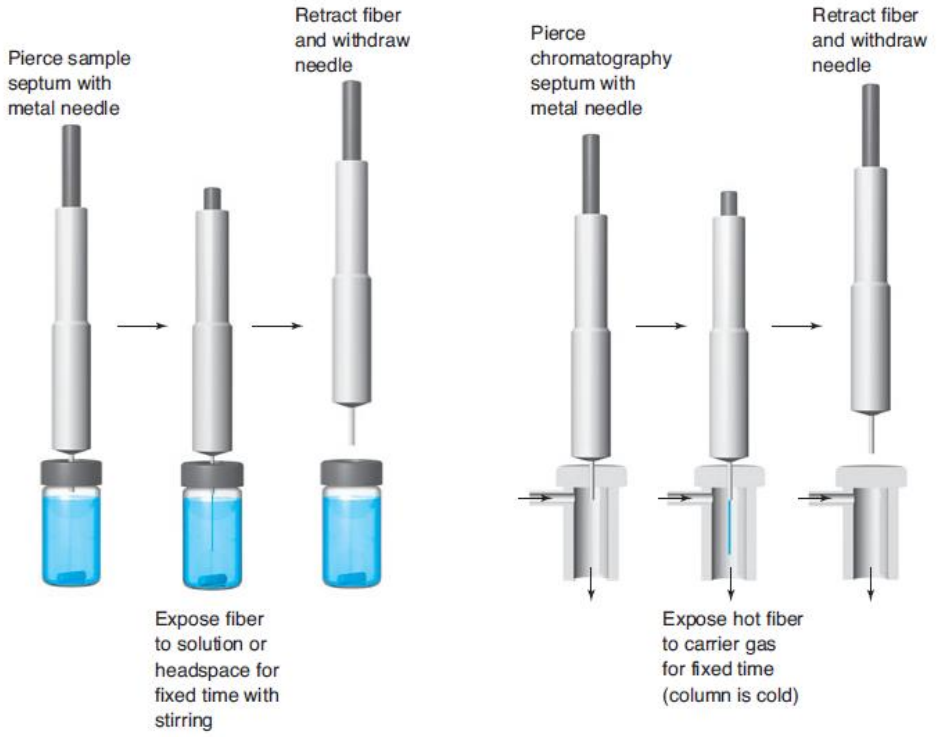

Figure 2.18: Schematic representation of SPME sampling procedure and subsequent desorption of extracted compounds from the SPME fibre in a GC injector (Reproduced from [69]).

PDMS is however a non-polar polymer and therefore its performance for sampling polar compounds is less satisfactory. For this reason, other phases have been devised; they include polyetheleneglycol (PEG) and polyacrylate, suitable for polar compounds, as well as the so called 'mixed phases' that incorporate, in addition to the polymer, adsorbents such as divinylbenzene or carboxen, thus giving them a much wider working polarity range. The two common sampling modes for SPME are immersion or headspace (HS-SPME) extraction, respectively. In immersion mode the fibre is inserted directly into the sample and the analytes partition from the sample matrix into the fibre coating. This mode is preferable for less volatile compounds which do not easily migrate into the headspace of the sample. The disadvantage of this, however, is that non-volatiles and/or particulate matter may be deposited on the fibre, significantly shortening its lifetime as a result of the subsequent exposure to heat in the injector [70]. HS-SPME is therefore most often used, especially in flavour and aroma analysis, since it is more representative of the particular aroma that will be perceived when consuming the product. In addition, the fibre is known to last much longer using this mode. SPME (and HS-SPME) has been successfully

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applied for sample enrichment for a large number of compounds in the food and beverage industry, including, wine, fruits, beverages, etc. [71].

2.3.4.2. Stir bar sorptive extraction (SBSE)

SBSE, first described by Baltussen et al. [72], is very similar to SPME and also employs PDMS as extraction phase. In this case a magnetic stir bar encapsulated in glass is coated with PDMS. The most marked difference between the two is that the PDMS coating on the stir bar is much thicker than that of SPME, thereby providing a much higher volume of the extraction phase. This increases sampling capacity significantly, and therefore much lower detection limits can be achieved by SBSE. The sampling process is similar to SPME: the stir bar can be either immersed in the sample while being stirred, or suspended in the headspace for sampling. Desorption, however, is performed somewhat differently in terms of hardware, although the principle is identical. A thermal desorption system coupled to a PTV injector is needed in order to desorb the analytes from the stir bar while the injector is at low temperature (typically -100^{o}C), followed by very rapid heating of the injector for sample introduction. One drawback of SBSE is that stir bars are mainly available with a PDMS phase, rendering the technique less useful for highly polar compounds. Examples of the use of SBSE in the analysis of food and beverages include analysis of aroma compounds in wine [73] and grape derived products, HS-SBSE of wine volatiles [74] and a range of other products [75].

2.3.4.3. The sample enrichment probe (SEP)

The SEP, developed by Burger et al. in 2006 [76], combines two important advantages SPME and SBSE, respectively. As with SBSE, the technique also makes use of a large amount of PDMS and, as is the case with SPME, no additional hardware is required for sample introduction (although some GC's do require slight modification of the injector). The SEP consists of a glass rod coated at the tip with a PDMS sleeve. Sampling is performed in the headspace, typically using long extraction times (> 10h) to ensure that equilibrium is reached. SEP has been successfully applied for, amongst other samples, the analysis of honeybush tea [18-20].

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Chapter 3

Analysis of honeybush tea (*Cyclopia* spp.) volatiles by comprehensive two-dimensional gas chromatography using a novel single-stage thermal modulator*

**This chapter is in preparation for publication*

Stellenbosch University https://scholar.sun.ac.za

Analysis of honeybush tea (*Cyclopia* spp.) volatiles by comprehensive two-dimensional gas chromatography using a novel single-stage thermal modulator

Abstract

The applicability of comprehensive two-dimensional gas chromatography (GC×GC) using a novel single-stage thermal modulator was explored for the analysis of honeybush tea (*Cyclopia* spp.) volatile compounds. Headspace solid phase micro-extraction (HS-SPME) was used in combination with GC×GC separation on a non-polar × polar column set with flame ionization (FID) and quadrupole mass spectrometry (qMS) detection for the analysis of fermented *C. maculata*, *C. subternata* and *C. genistoides* tea infusions of two harvest seasons. Method optimisation entailed evaluation of the effect of several experimental parameters on the performance of the modulator, as well as the choice of columns in both dimensions and HS-SPME conditions. 69 volatile compounds were identified by co-injection of reference standards, while 2 additional compounds were tentatively identified based on mass spectral and retention index data. The majority of the compounds were common to all three species, however principle component analysis (PCA) of the GC×GC-FID data showed clear differentiation between the species. Especially the volatile composition of *C. maculata* samples differed significantly from the other two species for the same harvest season, which was in agreement with sensory results for the same samples. It is noteworthy that due to the highly reproducible separations obtained using the single-stage thermal modulator, multivariate analysis of the 2-dimensional GC×GC-FID data was simplified. The results demonstrate both the complexity of honeybush volatile profiles, and the potential of GC×GC separation in combination with suitable data analysis techniques for the investigation of these compounds. The developed method therefore offers a fast and cheap methodology for the profiling of honeybush tea volatiles, and as such shows promise for further research focusing on the relationship between sensory properties and volatile composition of these products.

3.1. Introduction

Comprehensive two-dimensional gas chromatography (GC×GC) has found widespread application in the analysis of complex mixtures of volatile and semi-volatile compounds, such as petrochemical samples, natural products, environmental and food and beverage samples [1,2]. This powerful separation technique offers high resolving power, a consequence of the separation of analytes on two columns with different stationary phases [3–5]. The two columns are connected by a modulator which sequentially traps, focuses and re-injects analytes eluting from the first dimension (^{1}D) column onto the second dimension (^{2}D) column. Improved sensitivity due to refocusing of analytes in the modulator and the much higher resolving power of the technique greatly improves compound identification in complex mixtures.

The performance of GC×GC clearly depends on the operation principle of the modulator. A range of modulators have been developed, which can be classified broadly into valve-based (diaphragm valves or flow-modulation) and thermal (heater based or cryogenic) modulators [7]. Currently, the majority of commercial GC×GC systems utilise cryogenic modulation, although flow-modulation approaches are gaining popularity due to their relative simplicity and reduced operating costs. Cryogenic modulators provide excellent performance for most analytes, but they are relatively expensive and operation is costly due to the high demand for cryogenic agents such as liquid nitrogen. On the other hand, failure of heater-based modulators to effectively trap highly volatile compounds and the limited volatility range of these systems have hampered their more widespread use [8].

Recently, Górecki and co-workers reported a single-stage thermal modulator that offers consumable-free operation [9-11]. The design of this modulator was based on the original idea of Liu and Philips [12], but instead of using a painted fused-silica capillary, the modulator is based on a stainless-steel capillary trap coated with polydimethylsiloxane (PDMS). The key factor responsible for the successful operation of this modulator is sufficient restriction of the carrier gas flow in the modulator during the desorption step to prevent analyte breakthrough. This is achieved by flattening the trapping capillary and installing a restrictor capillary (deactivated fused silica) before the modulator. Desorption is performed through pulsed resistive heating of the trapping capillary with a capacitive discharge power supply. The restriction of the gas flow occurs due to the fact that the viscosity of carrier gas increases with the increase in temperature caused by the capacitive discharge. Rapid heating of the modulator during desorption creates a significant pressure drop across the capillary which momentarily stops the carrier gas flow from the first dimension column [9]. Following desorption, cooling of the trap to the oven temperature is facilitated by passive or active cooling by ambient air.

In this study, the application of this novel single-stage modulator was evaluated for the analysis of honeybush tea volatile compounds. Honeybush (*Cyclopia* spp.) is an indigenous herbal tea from South Africa which is well known for its characteristic sweet, and slightly honey-like, aroma [13]. The popularity of honeybush tea stems not only from its sweet taste and aroma, but also from its reputation as a healthy beverage, primarily associated with its content of flavonoids and xanthones [14]. Honeybush tea is produced from the leaves and stems of *Cyclopia* species which are endemic to the fynbos biome in the Western and Eastern Cape provinces of South Africa. To date more than 20 species have been described, although only *C. genistoides* and *C. subternata*, which are cultivated, and *C. intermedia*, which is wild harvested, are used for the production of honeybush tea. With

current demand exceeding the supply, *C. maculata*, *C. longifolia* and *C. sessiliflora* are being explored for honeybush tea production [13].

Taste and aroma are the important sensory characteristics used to evaluate the quality of tea [15]. These characteristics are determined by the chemical composition of the sample - non-volatile compounds are associated with taste and volatile compounds with aroma [16]. The volatile composition of honeybush tea has been studied extensively by Le Roux et al. [17-19] by means of gas chromatography-olfactometry (GC-O) and gas chromatography-mass spectrometry (GC-MS). In total, 255 volatile compounds were identified in unfermented and fermented infusions of *C. intermedia, C. subternata, C. genistoides* and *C. sessiliflora*, with terpenoids being the major chemical class, followed by aldehydes, esters, hydrocarbons and ketones. Forty-six of the identified compounds were established as being odour-active by GC-O [19]. Honeybush tea thus represents a complex sample ideally amenable to GC×GC separation. In the current work, the volatile composition of honeybush tea produced from three different *Cyclopia* species of two production years were analysed by GC×GC using the single-stage thermal modulator. Multivariate statistical analysis was used to ascertain differences of the volatile profiles among *Cyclopia* species and the relationship between volatile composition and sensory properties for the same samples.

3.2. Materials and methods

3.2.1. Chemical and materials

Fermented honeybush tea leaves used for method optimisation were supplied by the Department of Food Science (Stellenbosch University, SA). Solid phase micro-extraction (SPME) fibres (100 µm polydimethylsiloxane (PDMS), 65 µm PDMS/divinylbenzene (PDMS/DVB), 50/30 µm PDMS/CAR (carboxen), 50/30 µm PDMS/CAR/DVB, 85 µm CAR/PDMS, 85 µm polyacrylate (PA) and 60 µm polyethylene glycol (PEG)) and the SPME manual holder were purchased from Supelco (Bellefonte, PA, USA). Sodium chloride (NaCl) and a mixture of C_7-C_{40} linear hydrocarbons used for determination of the retention indices were purchased from Sigma Aldrich (St. Louis, MO, USA). Ascorbic acid was purchased from Hopkin & William (Johannesburg, SA) and reference standards were purchased from Fluka or Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA), or synthesised as detailed elsewhere [19]. A standard solution containing all reference standards at varying concentrations was prepared in dichloromethane (Sigma-Aldrich) [19]. Deionised water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA).

The plant material used to prepare the infusions was obtained from commercial processors or was prepared by laboratory-scale processing at the Post-Harvest and Wine Technology Division of ARC Infruitec-Nietvoorbij (Stellenbosch, SA) during the 2010 and 2013 harvesting seasons [20]. Each of the two sets of samples consisted of three different *Cyclopia* species, *C. genistoides*, *C. subternata* and *C. maculata*. Each species in each set was represented by 5 samples of fermented plant material. Details of the processing for laboratory-scale production have been described elsewhere [20].

3.2.2. Sample preparation

3.2.2.1. Preparation of tea infusion used for method optimisation

The same fermented plant material was used for all method optimisation experiments. Boiled deionised water (100 mL) was added to 1.25 g of honeybush tea leaves in a flask. The flask was covered with aluminium foil and the tea was infused for 5 min with occasional (manual) swirling. The hot solution was then filtered into a flask containing 90 mg ascorbic acid through a pre-washed (100 mL boiled deionised water) filter paper. The extract was allowed to cool to room temperature before SPME extraction. A fresh infusion was prepared for each set of optimisation experiments.

3.2.2.2. Samples used for sensory analysis and multivariate statistical analysis based on GC×GC data

Infusions were prepared as outlined by Theron et al. [20]. Boiled deionised water (900 g) was added to 11.25 g tea leaves, infused for 5 min and strained into a 1 L stainless steel thermos flask. The infusions were then subjected to sensory analysis at the Department of Food Science (Stellenbosch University, SA). Infusions (ca. 100 mL) were served in white porcelain mugs covered with plastics lids to prevent loss of volatiles. Measures were furthermore taken to keep the infusion temperature constant, *inter alia* by using temperature-controlled (65°C) scientific water baths (SMC, Cape Town, SA) during the serving of infusions to panellists [20]. Portions of the tea infusions were cooled and kept frozen at -4°C until GC×GC analysis.

3.2.3. Headspace-SPME extraction procedure

SPME fibres were conditioned as recommended by the manufacturer prior to use. Six fibres with different coatings (detailed in section 3.2.1) were evaluated. 10 mL of each infusion was transferred to a 20 mL vial containing 2 g NaCl (previously heated to 100°C and cooled to room temperature to remove moisture) and a PTFE stir bar. The vials were capped immediately and the fibre was exposed to the headspace of the sample. Extraction was performed at room temperature (21±2°C) using a stirring rate of 1000 rpm for 30 min and 1 hr for GC×GC-FID and GC×GC-qMS analyses, respectively. Fibres were pre-conditioned in a 250°C GC injection port for 30 min each day and blank analyses were performed daily to verify that the system and the fibre remained contaminant-free. The final method utilised the PDMS/DVB fibre with the same conditions as outlined above.

3.2.4. Instrumentation

All analyses were performed on an in-house built GC×GC system consisting of an Agilent 6890 GC (Agilent Technologies, Palo Alto, CA, USA) equipped with an FID, a 5973 Agilent mass selective detector (MSD) and a programmed temperature vaporizing (PTV) injector (Gerstel, Mulheim a/d Ruhr, Germany). The instrument was retrofitted with the single-stage thermal modulator (**Figure 3.1**). Modulation was achieved using a custom-made capacitive discharge power supply [10].

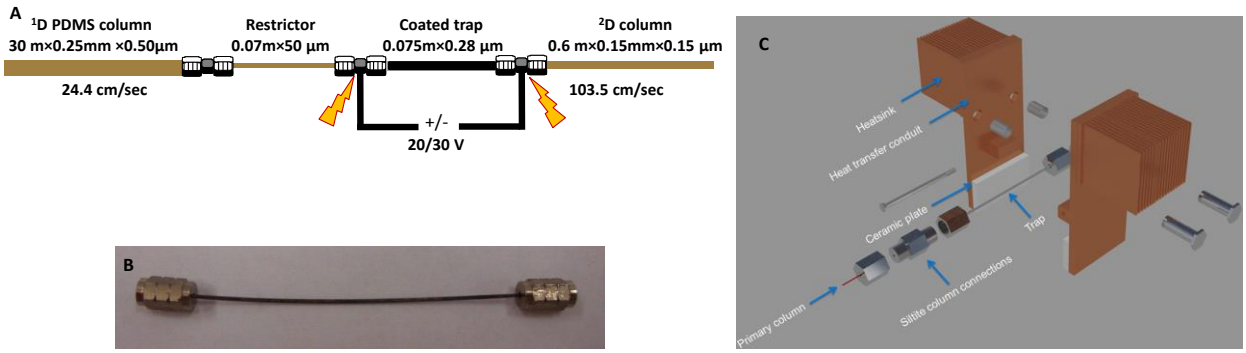

Figure 3.1: Overview of the single-stage thermal modulator used in this study. (**A**) Schematic illustration of the columns, restrictor and trap used, (**B**) photograph of the trap, and (**C**) shows the placement of the trap between two ceramic plates which are passively cooled by exposure to the passive heatsinks above the GC oven (reproduced from [10]).

3.2.5. Instrumental conditions

3.2.5.1. GC×GC optimisation experiments

Hydrogen (99.99% purity) was used as carrier gas for all experiments. The following conditions were used for the initial experiments: An initial flow rate of 2.05 mL/min and a constant inlet pressure of 152 kPa. The initial oven temperature was 40°C, held for 2 min, followed by a temperature ramp at 5.5°C/min to 240°C, held for 5 min. Analytes were desorbed in the PTV at 240°C for 5 min in splitless mode (splitless time 1.5 min). The FID temperature was set to 250°C (air = 450 mL/min, H_2 = 40 mL/min, make up gas N_2 = 45 mL/min) and an acquisition rate of 100 Hz was used.

For evaluation of the SPME fibres a non-polar × polar column set was employed: 30 m × 0.25 mm × 0.50 µm HP-5MS (J&W Scientific, Folsom, CA) and 0.6 m × 0.15 mm × 0.15 µm Stabilwax (Restek, Penn Eagle Park, CA, USA) columns were used in ^{1}D and ^{2}D, respectively. Two second dimension columns with different polarities, 50% phenyl/50% PDMS (Rxi-17Sil MS, 0.6 m × 0.15 mm × 0.15 µm, Restek) and trifluoropropyl methyl polysiloxane (Rtx-200, 0.6 m × 0.15 mm × 0.15 µm, Restek) were also evaluated for the non-polar × polar configuration. For evaluation of a polar × non-polar column combination, a 30 m × 0.25 mm × 0.25 µm DB-WAXETR column (J&W Scientific) was used in ^{1}D and a 0.6 m × 0.15 mm × 0.15 µm Rxi-5ms column (Restek) in ^{2}D. For the optimal column combination (HP-5MS × Stabilwax), the optimisation of carrier gas flow was evaluated at head pressures of 75, 100 and 151 kPa. Oven temperature ramping rates of 2, 3 and 5.5°C/min were evaluated.

3.2.5.2. Optimised GC×GC method

The optimised GC×GC configuration consisted of 30 m × 0.25 mm × 0.50 µm HP-5MS (J&W Scientific) and 0.6 m × 0.15 mm × 0.15 µm Stabilwax (Restek) columns in the first and second dimensions, respectively. Analyses were performed at a constant column head pressure of 100 kPa. The modulation period was 5 s, and discharge voltages of 20 V and 30 V were used. Analytes were desorbed for 2 min in the GC inlet at 240°C. The first set of samples were injected in split mode (split ratio 1:10) and the second set and GC×GC-

quadrupole mass spectrometry (qMS) analyses were performed in splitless mode (splitless time 1.5 min). The oven was held at 40°C for 2 min, ramped at 3°C/min to 240°C and held for 5 min at the final temperature.
For GC×GC-qMS analyses, the column head pressure was lowered to 45 kPa to compensate for the vacuum outlet. The transfer line, analyzer and ion source temperatures were set to 240, 150 and 230°C, respectively. MS spectra were acquired at a scan rate of 22 Hz with a mass range from 50 to 200 amu. A solvent delay of 5 min was used for liquid injections of the standards. NIST05 (National Institute of Standards and Technology, Gaithersburg, MD, USA) and Wiley 2.75 (Wiley, New York, USA) mass spectral libraries were used for tentative identification of compounds.

3.2.6. Data processing

3.2.6.1. Creation of contour plots

GC×GC-FID raw chromatograms were exported from ChemStation software (MSD ChemStation D.01.00 SDK, Agilent) as .csv (comma-separated values) files. Contour plots were then created in MATLAB (R2008a, The MathWorks Inc., Natick, MA, USA) using built-in functions.

3.2.6.2. Multivariate data analysis for GC×GC

The .csv files were imported into MATLAB (Version R2012b, The MathWorks Inc.) using built-in functions for further data processing on a PC (64 bit Intel® Core™ i7 CPU with 3.40 GHz and 24 GB of RAM). All second dimension chromatograms were baseline corrected using a weighted least squares baseline function (Eilers' method based on a Whittaker filter) [22] of the PLS Toolbox for MATLAB (PLS Toolbox 7.5, Eigenvector Research Inc. USA). Each raw chromatogram and the corresponding baseline corrected chromatogram were visually compared to ensure that no artefacts were introduced during baseline correction. Peak picking of two-dimensional GC×GC-FID peaks was performed using the extrema2.m function, which searches for all the maxima (peaks) in a matrix, via columns, rows and crossing diagonals [23]. An in-house developed function was used to automatically integrate each ^{2}D peak slice and subsequently sum the obtained area values. The automated peak integration was monitored visually by plotting contour plots of each peak for multiple samples, including its integration range. Due to the good reproducibility of the two-dimensional chromatograms no peak alignment was necessary and the integration of only a few peaks had to be manually corrected. The obtained peak table was then pareto scaled and mean centered prior to principle component analysis (PCA).

3.2.7. Descriptive sensory analysis

Descriptive sensory analysis (DSA) was used to determine the aroma profile of honeybush infusions [24]. The sensory panel consisted of ten female assessors who had extensive experience in the assessment of honeybush tea quality. Training sessions were firstly conducted to generate the suitable aroma descriptors associated with different *Cyclopia* species. The list of 68 aroma attributes, based on the sensory profiles of six *Cyclopia* species as generated in [20], was used as a basis to determine the 22 descriptors

associated with the aroma profile of *C. genistoides, C. subternata* and *C. maculata.* Once the panellists had a good understanding of the product, the testing phase started. The samples were labelled with three-digit codes and presented in randomised order. The panellists rated the intensities of the aroma attributes on an unstructured line scale (0 – 100), using *Compusense® five* software program (Compusense, Guelph, Canada). The samples were analysed in triplicate in three consecutive sessions to test judge reliability. All analyses were conducted in booths fitted with controlled lighting.

The data from all assessors for each individual sample were collected and analysed. The performance of the panel was monitored using *Panelcheck* software (Version 1.4.0, Nofima Mat, Ås, Norway). SAS® software (Version 9.2; SAS Institute Inc., Cary, USA) was used to subject the data to test-retest analysis of variance (ANOVA). The residuals were tested for non-normality by using the Shapiro-Wilk test and outliers were identified and removed in the event of significant non-normality ($p \leq 0.05$). Principal component analysis (PCA) and discriminant analysis (DA) plots were produced using XLStat (Version 2013.5.07, Addinsoft, New York, USA) to provide a graphical representation of the relationship between the samples and their attributes.

3.3. Results and discussion

3.3.1. Evaluation of modulator performance

Effective modulation requires trapping and focusing of analytes eluting from the ^{1}D column, followed by efficient re-injection as short focused plugs onto the ^{2}D column. A novel single-stage thermal modulator consisting of a coated stainless steel trapping capillary was used in this study [10,11]. Trapping of analytes is achieved by passive cooling of the trap using ceramic pads connected to heat sinks exposed to ambient temperature outside the GC oven (**Figure 3.1**). Re-injection is achieved by resistive heating of the trap by capacitive discharge using an external power supply which is timed by the FID clock. For the thermal modulator used here, the temperature of the trapping capillary has to be sufficiently low during the trapping phase to prevent analyte breakthrough, while the desorption temperature has to be sufficiently high during the injection step (this is controlled in practice by the voltage applied during the short discharge step).

To verify the effectiveness of the ceramic plates to maintain the trapping capillary at a lower temperature than the oven, a thermocouple was mounted between the ceramic pads during a temperature programmed analysis to measure the temperature of the pads relative to the oven temperature. Measurements were taken at 1 min intervals, and the results are presented in the Supporting Information (**Figure S3.1**). The results confirmed that the temperature between the plates was consistently lower than the oven temperature throughout the analysis, reaching a maximum difference of 64.5°C when the oven reached its maximum value. Based on this observation, it can be concluded that the heat sinks are effective at cooling the ceramic plates, and as a consequence the trapping capillary clamped between them. This difference in temperature should be sufficient to trap analytes effectively in the absence of overloading effects. It is also interesting to note from this experiment (**Figure S3.1**) that cooling of the modulator to its initial temperature takes approximately 15 min. This is the minimum time that should be used between injections.

3.3.2. Method optimisation

3.3.2.1. Evaluation of SPME fibres

Honeybush tea has been shown to contain a wide range of volatile organic compounds of varying polarities and chemical properties, and spanning several orders of magnitude in concentration [17,18]. SPME was selected for this study because of the well-known benefits of this technique: it provides solvent free, selective extraction and pre-concentration of volatile analytes in a single step. SPME has shown good efficiency in extracting volatile compounds of a range of beverages [25,26]. Since the efficiency of the SPME extraction depends on the polarity, porosity and the volume of fibre-coating [27], six different SPME fibres (100 µm PDMS, 85 µm CAR/PDMS, 50/30 µm DVB/CAR/PDMS, 65 µm PDMS/DVB, 85 µm PA and 60 µm PEG) were evaluated for the extraction of honeybush volatiles. Less polar fibres like PDMS and CAR/PDMS are more suitable for extraction of volatile and semi-volatile compounds with low to medium polarity, whilst polar fibres such as PA and PEG may be used for the extraction of more polar compounds. DVB mixed fibres have unique selectivity and display affinity for volatile compounds of different polarities [28]. Of the six coatings tested, PDMS/DVB and CAR/DVB/PDMS extracted more compounds (refer to **Figure S3.2**), as has also been reported previously [28]. Differences were observed in the relative amounts of analytes extracted by each fibre. In the case of the DVB/CAR/PDMS fibre, extensive overloading of the trap was observed, as evidenced by the 'streaking` peak shapes in the second dimension (**Figure S3.2**). The PDMS/DVB fibre was therefore chosen for further analyses.

3.3.2.2. Selection of the column combination for GC×GC separation

Non-polar × polar column sets are most common in GC×GC, in part due to the higher 'orthogonality' offered by separation according to boiling point in the ^{1}D column followed by selective interaction in the ^{2}D column. For polar × non-polar combinations the contribution of partial vapour pressures also plays a role in ^{1}D separation, resulting in some compounds having strong retention correlation with a second dimension boiling point separation [8]. Nevertheless, polar × non-polar column sets have demonstrated excellent performance for particular samples like petroleum products [29]. The potential applicability of a polar × non-polar column set was therefore also investigated for honeybush tea volatiles. A comparison of contour plots obtained on PDMS × wax and wax × PDMS column combinations is shown in the Supporting Information, **Figure S3.3**. The non-polar × polar column combination indeed showed better utilisation of the two-dimensional separation space as well as less breakthrough from the trap, as is evident from less 'streaking'. This is likely due to the fact that more polar compounds, which are weakly retained on the non-polar trapping phase, also elute from the ^{1}D column at higher temperatures when a polar column is used in the first dimension. Subsequent method development was therefore performed using non-polar × polar column combination set.

For the more polar honeybush volatiles, wrap-around was observed. This phenomenon occurs on polar ^{2}D columns, where high retention in this dimension results in compounds eluting in subsequent modulation period(s). In an attempt to reduce wraparound of some of the major compounds (highlighted in **Figure S3.4A-C)**, alternative ^{2}D columns of lower polarity were evaluated: trifluoropropyl methyl polysiloxane (Rtx-200, 0.6 m × 0.15 mm × 0.15 µm) and 50% phenyl/50% PDMS (Rxi-17Sil MS, 0.6 m × 0.15 mm × 0.15 µm). With the least polar ^{2}D column (Rxi-17Sil MS, **Figure S3.4F**), wraparound was significantly reduced.

However, the lower retention of the majority of honeybush volatiles on this column also resulted in much worse overall separation and less effective utilisation of the two-dimensional separation space (**Figure S3.4C**). The PDMS × Stabilwax column combination was therefore chosen for further method optimisation. Increasing the modulation period is an obvious choice to reduce wraparound, although this is associated with a loss of ^{1}D resolution due to insufficient sampling of first-dimension peaks (see further).

3.3.2.3. Optimisation of the carrier gas flow and temperature programming

One of the challenges in GC×GC method optimisation is that most experimental parameters are interrelated [30]. Since the column head-pressure determines the carrier gas flow rates in both columns, GC×GC analyses are typically carried out at carrier gas velocities close to the optimum for the ^{1}D column, but considerably above the optimal velocity in the ^{2}D column for maximum speed in this dimension [31]. It has been suggested that acceptable linear velocities for hydrogen in ^{1}D and ^{2}D columns are approximately 20 cm/sec and 100-130 cm/sec, respectively [8]. Optimisation of the carrier gas flow rate was performed according to these guidelines using constant column head pressures of 151, 100 and 75 kPa, which produced initial carrier gas flow rates of 2.05, 1.06 and 0.81 mL/min, respectively. These experiments were performed at a constant ramp rate of 5.5°C/min. The average linear velocities in both dimensions were calculated according to the method of Shellie et al. [32] using a Hewlett-Packard flow calculator. A head pressure of 151.7 kPa produced (initial) linear velocities of 39.4 cm/sec and 190.8 cm/sec in ^{1}D and ^{2}D, 100 kPa produced linear velocities of 24.4 and 103.5 cm/sec, and 75 kPa produced linear velocities of 20.42 and 79.6 cm/sec, respectively. **Figures 3.2A-C** illustrate the effect of flow rate on the separation. At high flow rates, resolution in ^{1}D was compromised, whereas at a low flow rate resolution in ^{2}D decreased since the analytes now elute at higher temperatures. An intermediate flow rate of 1.06 mL/min was therefore selected as optimal, resulting in a carrier gas linear velocity in ^{2}D within the range of 100-130 cm/sec [8].

The GC oven temperature program is known to have a major influence on the separation and was therefore optimised. In GC×GC, slow ramping rates generate broad first dimension peaks, which facilitate sufficient sampling rates of first-dimension peaks to satisfy sampling requirements [33]. In the case of the thermal modulator used here, broader ^{1}D peaks are also beneficial in the sense that the amount of analyte trapped during each modulation step will be reduced, thereby minimising the risk of breakthrough occurring during the trapping step. On the other hand, a very slow ramp increases the analysis time significantly, and may produce slightly broader ^{2}D peaks. For these reasons this parameter should be optimised carefully [8]. Using a carrier gas flow rate of 2.06 mL/min, the oven temperature ramping rate was varied between 2°C/min and 5.5°C/min. **Figures 3.2D-F** show the 2D contour plots obtained for honeybush tea volatiles at ramping rates of 5.5°C/min, 3°C/min and 2°C/min. Using a ramping rate of 5.5°C/min, separation in the first dimension was clearly compromised due to both decreased resolution and increased under-sampling. A ramping rate of 3°C/min was chosen as a compromise to maintain good resolution for acceptable analysis times and to reduce wraparound. This value is in agreement with literature, where ramping rates ranging from 1 – 3°C/min are often used [8].

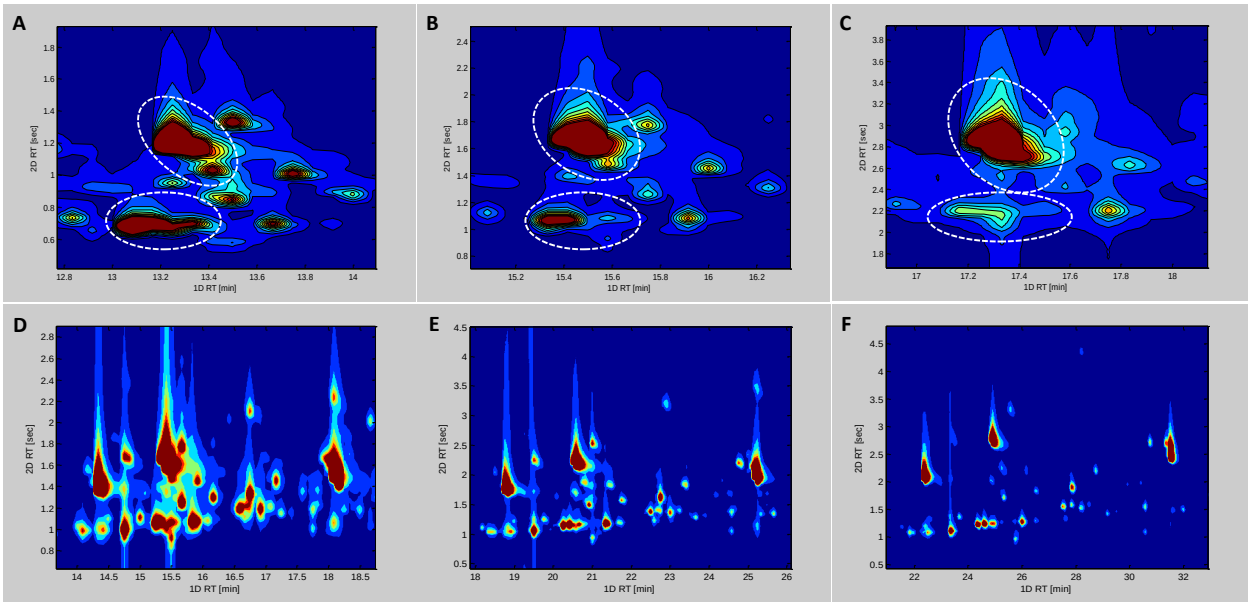

Figure 3.2: Illustration of the effect of carrier gas flow rate and GC oven temperature program on the GC×GC separation of honeybush tea volatiles. The effect of flow rate is shown for selected parts of the GC×GC-FID contour plots (**A**) 75 kPa (u_{1D}= 20.4 cm/sec, u_{2D}= 79.6 cm/sec), (**B**) 100 kPa (u_{1D} = 24.4 cm/sec, u_{2D} = 103 cm/sec), and (**C**) 151.7 kPa (u_{1D} = 39.4 cm/sec, u_{2D} = 191 cm/sec). The effect GC oven temperature ramping rate is illustrated in contour plots (**D**) 5.5°C/min, (**E**) 3°C/min and (**F**) 2°C/min. Analyses were performed on PDMS × Stabilwax column set; SPME fibre = PDMS/DVB. Other chromatographic conditions are as specified in **Section 3.2.5.1**.

3.3.2.4. Modulation temperature (discharge voltage) and modulation period

The modulation period of 5 s used in initial experiments was long enough to minimise wraparound, but short enough to minimise first dimension under-sampling, and was therefore used for all subsequent analyses. Trapped analytes in the modulator have to be re-injected as a narrow band onto the ^{2}D column. Re-mobilisation of analytes must be rapid to avoid band broadening, therefore the desorption temperature of the modulator has to be high enough to allow rapid volatilisation of analytes. In the case of the modulator utilised in this study, desorption is achieved by resistive heating of the trapping capillary using a single channel discharge voltage power supply. The discharge voltage bears a direct relationship with temperature and therefore determines the efficiency of re-mobilisation. Voltages of 20 and 30 V were used to evaluate the effect of discharge voltage on chromatographic separation; the corresponding contour plots are shown in **Figure 3.3**. At 20 V (**Figure 3.3A**), broad and deformed peaks shapes are observed due to slow release of trapped analytes. At 30 V (**Figure 3.3B**) well-defined ^{2}D peaks were mostly observed and the sensitivity was enhanced.

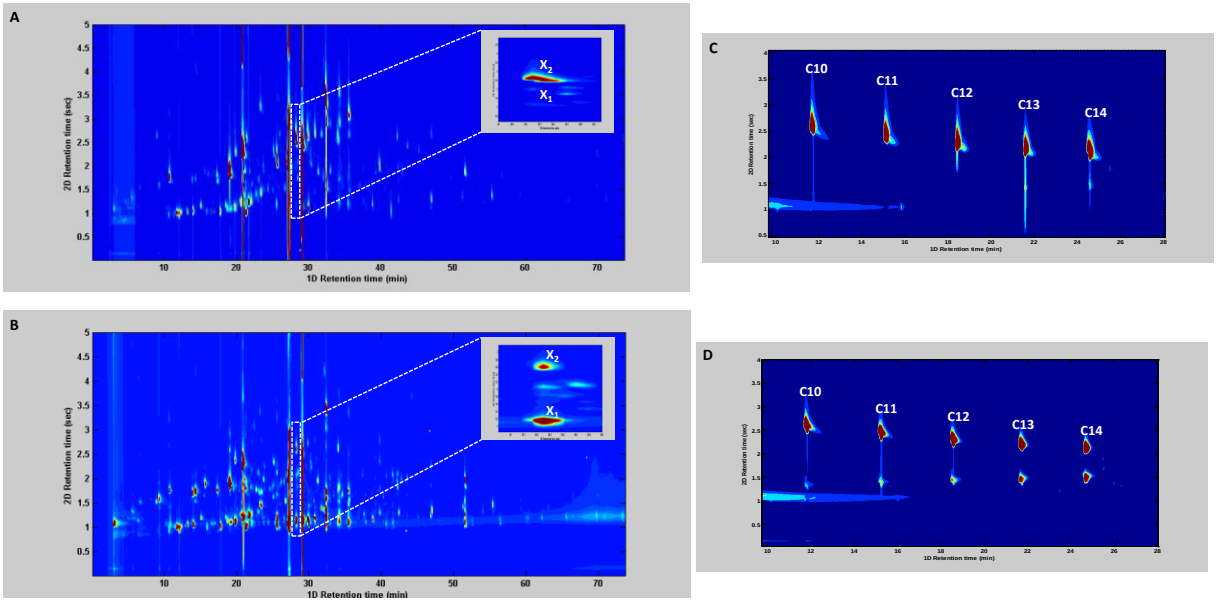

Figure 3.3: Illustration of the effect of the modulator discharge voltage on the GC×GC separation of honeybush tea volatile compounds: (**A**) 20 V and (**B**) 30 V discharge voltages. (**C**) and (**D**) show the analysis of C10-C14 linear alcohols at 20 V and 30 V, respectively. Analyses were performed using the optimised conditions (**Section 3.2.5.2**), except that the oven temperature ramping rate was 5.5°C/min for the alcohols.

However, the use of high discharge voltage is known to reduce the lifetime of the trapping capillary [11]. Furthermore, analyte breakthrough was observed at high discharge voltage as is evident by the detection of multiple peaks for some compounds. An example is illustrated in **Figures 3.3C** and **Figure 3.3D**, where polar standards were injected into the GC×GC system to investigate the breakthrough phenomenon. **Figure 3.3C** and **Figure 3.3D** show the contour plots for linear alcohols (C10-C14) analysed at 20 V and 30 V, respectively; breakthrough was much more severe at the higher discharge voltage. Although the temperature difference between the cooling ceramic plates and the GC oven has been shown to increase with the oven temperature (see **Figure S3.1**), breakthrough became significantly more pronounced for C12 to C14 linear alcohols which eluted at higher temperatures (**Figure 3.3D**) and were therefore not effectively trapped [11].

Because the traps used in this study were custom made, some variation in the performance between traps was observed. For example the trap used for the analysis of the first set of samples (set 1), 20 V was found to be optimal based on reduced breakthrough observed for this trap. For sample set 2, where a different trap was used, 30 V was found to be optimal due to lower breakthrough and better peak shapes at the higher voltage.

3.3.3. Identification of honeybush tea volatiles

Two sets of honeybush tea samples from 2010 and 2013 harvests were subjected to GC×GC-FID analysis. Each set contained 5 samples each of *C. genistoides*, *C. maculata* and *C. subternata*. All samples were analysed in duplicate. The two sets of samples were found to differ significantly in the abundance of volatile compounds. To accommodate this observation, injection was done in split mode for the first set to minimise modulator overload,

whereas for the second set splitless injection was used to maximize the chemical information.

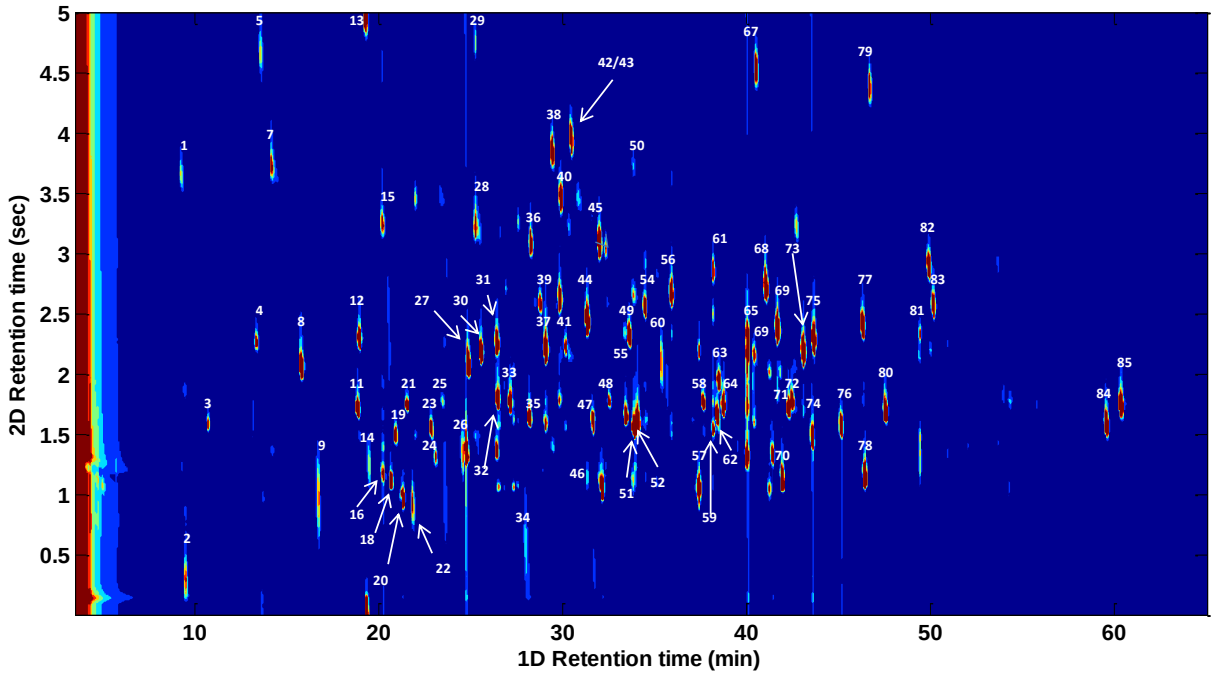

Figure 3.4: GC×GC-FID contour plot obtained for the separation of honeybush tea reference standards. The analysis was performed using optimised conditions (refer to **Section 3.2.5.2**). Peak numbers correspond to **Table 3.1**.

Identification was primarily based on comparison of retention times with reference standards and ^{1}D retention index values compared to NIST and PHEROBASE databases. A total of 69 compounds were identified using reference standards. These include 5-methylfurfural (**14**), identified here for the first time in honeybush tea. **Figure 3.4** shows the GC×GC-FID contour plot obtained for the analysis of the reference standards, and **Figure 3.5** shows representative contour plots obtained for each of the three *Cyclopia* spp. of the 2013 sample set. Peak numbers in these chromatograms correspond to **Table 3.1**. Most compounds were common to all three *Cyclopia* spp., with differences observed primarily in terms of their relative amounts.

From **Figure 3.5** it is clear that some of the major honeybush volatiles could not be identified using the available standards. In an attempt to identify some of these compounds, hyphenation to qMS was explored. Because of the reduced outlet pressure when using the MS, the column head pressure was lowered from 100 kPa to 45 kPa to obtain comparable retention times with the GC×GC-FID configuration [34]. The qMS was operated in scan mode with a mass range of 50-200 amu, resulting in a scan frequency of 22 Hz. This provided only about 2-5 spectra across each ^{2}D peak, much too low to accurately define the peaks or for quantitative analysis [33], but sufficient to identify methyl hexanoate and 6-methyl-5-hepten-2-one (highlighted in bold in **Table 3.1**) based on mass spectral matches greater than 80% and retention index data. The identity of the remaining major unknown compounds could not be ascertained as the MS spectral matches were poor.

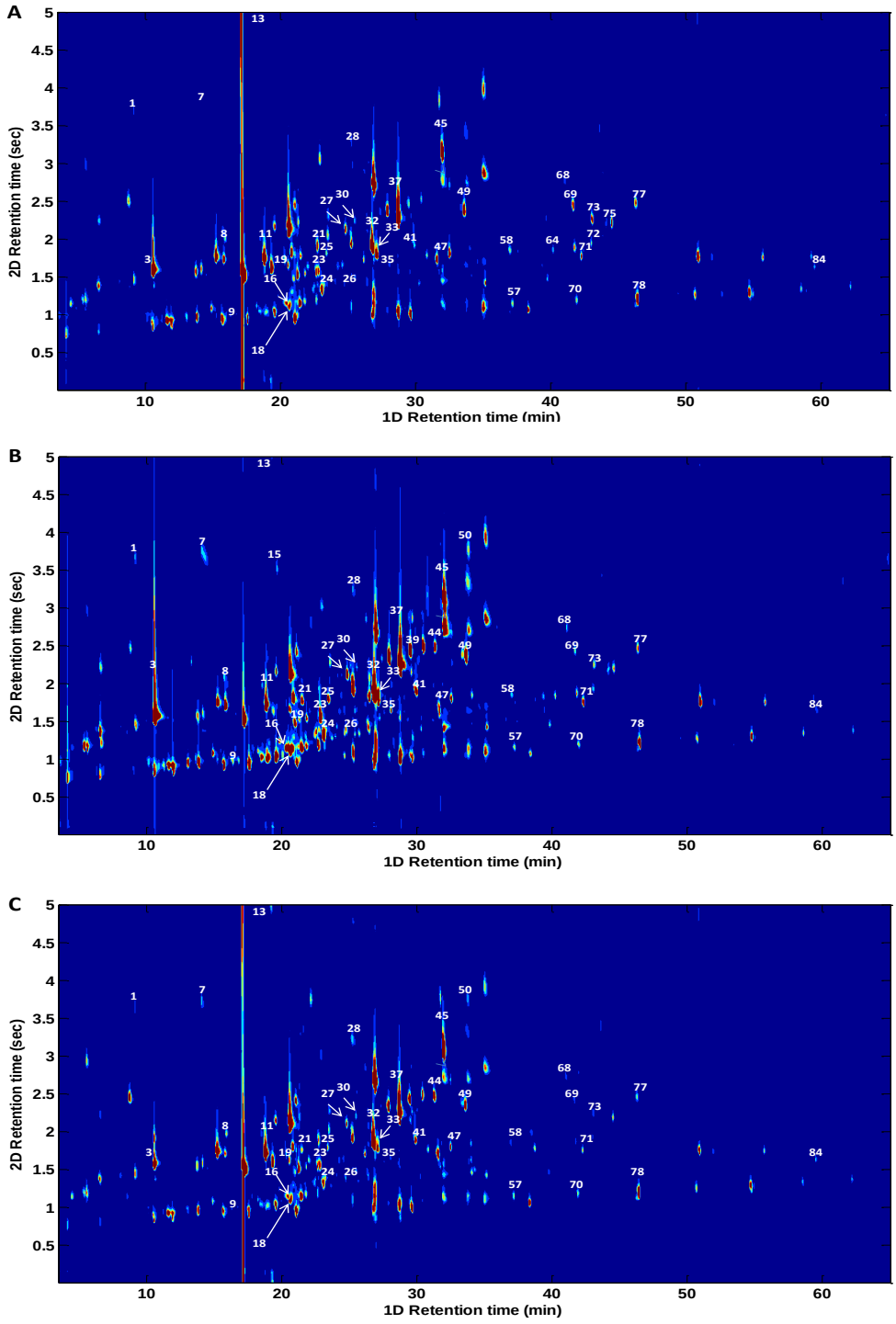

Figure 3.5: Representative GC×GC-FID contour plots obtained for the analysis of (**A**) *C. maculata*, (**B**) *C. subternata* and (**C**) *C. genistoides* samples of the 2013 harvest (set 2). Analyses were performed using optimised conditions (refer to **Section 3.2.5.2**). Peak numbers correspond to **Table 3.1** and **Figure 3.4**.

Figure 3.5 illustrates on the one hand the complexity of the honeybush tea volatiles, and on the other the performance of the single-stage thermal modulator for their comprehensive analysis. Some overloading is observed for high-level and polar compounds, yet overall

excellent resolution is obtained and good utilisation of the two-dimensional separation space is evident. The benefit of the two-dimensional separation is clear from **Figure 3.6**, which shows the raw FID data depicting five consecutive ^{2}D chromatograms and the corresponding part of the contour plot. In these slices compounds labelled **unknown 2** and **unknown 3** co-elute perfectly in the first dimension, but are separated on the polar ^{2}D column, whilst is the opposite for **unknown compound 1** and limonene (**23**), which co-elute in the second dimension but are separated in the first.

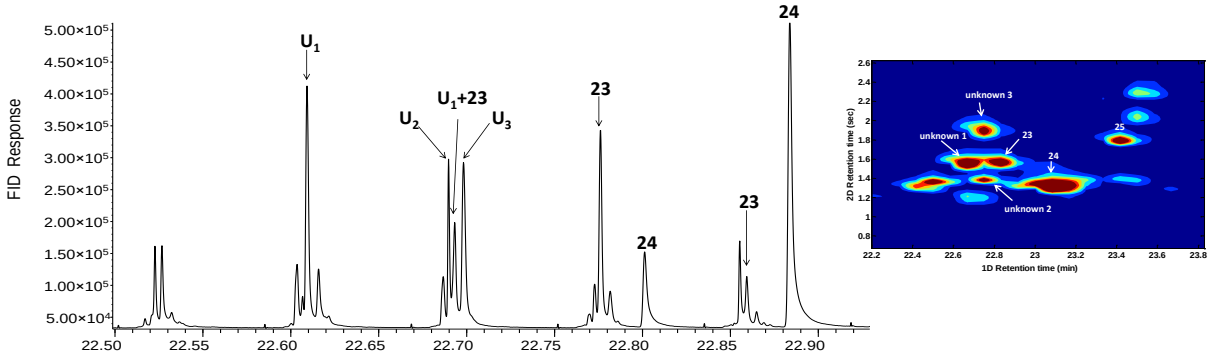

Figure 3.6: Zoomed in region of the raw GC×GC-FID chromatogram of *C*. *maculata* illustrating the resolution of the two-dimensional separation. Peak labels correspond to **Table 3.1, Figures 3.4** and and **Figure 3.5.** U_x = unknown.

For accurate identification of compounds and for comparison of volatile profiles between samples, it is essential to achieve very stable retention times in both dimensions. In GC×GC, shifts in ^{1}D retention times are caused by irreproducible injection and/or small variations in temperature and carrier gas flow [30], while shifts in ^{2}D are primarily caused by the modulator. To evaluate the repeatability of the thermal modulator used in this work, a *C*. *maculata* sample was analysed six times each on two consecutive days to determine intraday and interday repeatability. Eight compounds covering a wide range of retention in both dimensions were selected for this purpose (**Table 3.2**). The average relative standard deviation (RSD) for ^{1}D and ^{2}D retention times were 0.07% and 1.34%, respectively. The shifts in ^{1}D correspond to ± 1 modulation period, and are mainly due to the fact that manual injection was used. This also influenced the ^{2}D retention times to some extent as the analytes elute at slightly different temperatures due to slight discrepancies between the time of injection and starting each analysis. Despite this, however, the modulator showed excellent retention time reproducibility, which proved especially beneficial in the comparison of volatile profiles between species, as discussed in the next section.

Table 3.1: Volatile compounds identified in *Cyclopia* species by GC×GC-FID/qMS.

No.	Compound Name	RI_{cal}[a]	RI_{Lit}[a]	1st Set			2nd Set		
				GEN	MAC	SUB	GEN	MAC	SUB
1	1-Pentanol[b]	766	779	✓	✓	✓	✓	✓	✓
2	(*Z*)-2-Penten-1-ol[b]	772	783	×	×	×	×	×	×
3	Hexanal[b]	803	800	✓	✓	✓	✓	✓	✓
4	(*E*)-2-Hexenal[b]	854	854	×	×	×	✓	✓	✓
5	(*Z*)-3-Hexen-1-ol[b]	859	857	✓	✓	✓	✓	✓	✓
6	1-Hexanol[b]	867	867	✓	✓	✓	×	×	×
7	3-Methylbutanoic acid[b]	870	877	×	×	×	✓	✓	✓
	(*Z*)-4-Heptenal[b]	903	899	×	✓	×	✓	✓	✓
9	γ-Butyrolactone[b]	919	922	×	✓	✓	✓	✓	✓
10	**Methyl hexanoate**[c]	924	924	✓	✓	✓	×	×	×
11	6-Methyl-2-heptanone[b]	958	956	✓	✓	✓	✓	✓	✓
12	(*E*)-2-Heptenal[b]	960	961	×	×	×	✓	✓	✓
13	Benzaldehyde[b]	964	969	✓	✓	✓	✓	✓	✓
14	5-Methylfurfural[b]	969	978	✓	✓	×	✓	✓	✓
15	β-Pinene[b]	981	984	×	×	×	✓	✓	✓
16	1-Octen-3-ol[b]	982	983	✓	✓	✓	✓	✓	✓
17	**6-Methyl-5-hepten-2-one**[c]	985	985	✓	✓	✓	×	×	×
18	(6*Z*)-2,6-dimethyl-2,6-Octadiene[b]	990	990	×	×	×	✓	✓	✓
19	2-Pentylfuran[b]	994	993	✓	✓	✓	✓	✓	✓
20	Decane[b]	1002	1000	×	×	×	✓	✓	✓
21	Octanal[b]	1006	1007	✓	✓	✓	✓	✓	✓
22	2-Formyl-1-methylpyrrole[b]	1011	1010	✓	✓	✓	✓	✓	✓
23	Limonene[b]	1031	1031	✓	✓	✓	×	×	×
24	(*Z*)-β-Ocimene[b]	1033	1037	×	×	×	✓	✓	✓
25	(*E*)-3-Octen-2-one[b]	1042	1040	×	×	×	×	×	×
26	γ-Terpinene[b]	1064	1063	✓	✓	✓	✓	✓	✓
27	2,6,6-Trimethylcyclohexen-2-one[b]	1065	1042[d]	✓	✓	✓	✓	✓	✓
28	1-Octanol[b]	1073	1074	×	×	×	✓	✓	✓
29	Acetophenone[b]	1073	1078	×	×	×	×	×	×
30	*trans* Linalool oxide (furanoid)[b]	1079	1076	✓	✓	✓	✓	✓	✓
31	*cis* Linalool oxide (furanoid)[b]	1094	1091	✓	✓	✓	✓	✓	✓
32	2-Nonanone[b]	1095	1093	✓	✓	×	✓	✓	✓
33	Nonanal[b]	1108	1109	✓	✓	✓	✓	✓	✓
34	2-Phenylethanol[b]	1123	1127	×	×	×	✓	✓	✓
35	Methyl octanoate[b]	1127	1128	✓	✓	✓	×	×	×
36	Isophorone[b]	1128	1030	×	×	×	×	×	×
37	(*E*)-3-Nonen-2-one[b]	1144	1144	✓	✓	✓	✓	✓	✓
38	4-Ketoisophorone[b]	1150	1152	×	×	×	×	×	×
39	(*E*,*Z*)-2,6,-Nonadienal[b]	1158	1159	✓	×	✓	×	×	×
40	4-Vinylanisole[b]	1159	1160	×	×	×	×	×	×

Table 3.1 (*continued*)

No.	Compound Name	RI_{cal} [a]	RI_{Lit} [a]	1st Set			2nd Set		
				GEN	MAC	SUB	GEN	MAC	SUB
41	(*E*)-2-Nonenal[b]	1164	1165	✓	✓	×	×	×	×
42	Benzyl acetate[b]	1170	1172	✓	✓	×	×	×	×
43	Propiophenone[b]	1170	1176	✓	✓	×	×	×	×
44	Terpinen-4-ol[b]	1186	1181	✓	✓	✓	✓	✓	✓
45	α-Terpineol[b]	1198	1203	✓	✓	✓	✓	✓	✓
46	Dodecane[b]	1202	1200	✓	✓	✓	×	×	×
47	Decanal	1207	1210	×	×	×	✓	✓	✓
48	(*Z*)-3-Hexenyl-2-methyl butanoate[b]	1227	1231	✓	✓	×	×	×	×
49	β-Cyclocitral[b]	1230	1234	✓	✓	✓	×	×	×
50	Nerol[b]	1235	1236	✓	✓	✓	×	×	×
51	Methyl nonanoate[b]	1237	1227	✓	✓	✓	×	×	×
52	(*Z*)-3-Hexenyl isovalerate[b]	1240	1240	✓	✓	✓	×	×	×
53	Geraniol[b]	1244	1255	✓	✓	✓	×	×	×
54	Neral[b]	1248	1248	✓	✓	✓	✓	✓	✓
55	p-Anisaldehyde[b]	1265	1270	×	×	×	×	×	×
56	Geranial[b]	1277	1277	✓	✓	✓	✓	✓	✓
57	2-Undecanone[b]	1307	1296	×	×	×	✓	✓	✓
58	Geranyl formate[b]	1312	1305	✓	✓	✓	✓	✓	✓
59	Undecanal[b]	1323	1310	✓	✓	✓	✓	✓	✓
60	(*E*,*E*)-2,4-Decadienal[b]	1323	1319	×	×	×	×	×	×
61	Methyl decanoate[b]	1325	1326	×	×	✓	×	×	×
62	Theaspirane isomer 1	1328	1313	×	×	×	×	×	×
63	Hexyl tiglate[b]	1330	1337	✓	✓	✓	✓	✓	✓
64	*cis*-3-Hexenyl-(E)-2-methyl-2-butenoate[b]	1335	1312[d]	✓	✓	✓	✓	✓	✓
65	α-Cubebene[b]	1361	1362	✓	✓	✓	×	×	×
66	Eugenol[b]	1361	1373	×	×	×	×	×	×
67	Nonan-4-olide[b]	1372	1377	×	×	×	×	×	×
68	Butyl benzoate[b]	1382	1389	✓	✓	✓	✓	✓	✓
69	Geranyl acetate[b]	1396	1381	×	×	×	✓	✓	✓
70	(*E*)-β-damascenone[b]	1402	1400	✓	✓	✓	✓	✓	✓
71	Dodecanal[b]	1409	1414	✓	✓	✓	✓	✓	✓
72	6,10-Dimethyl-2-undecanone[b]	1413	1410	✓	✓	✓	×	×	×
73	(*E*)-β-Damascone[b]	1428	1409	✓	✓	✓	✓	✓	✓
74	(*E*)-Caryophyllene[b]	1439	1419	✓	✓	✓	×	×	×
75	α-Ionone[b]	1441	1426	✓	✓	✓	✓	✓	✓
76	α-Humulene[b]	1474	1477	×	×	×	×	×	×
77	(*E*)-β-Ionone[b]	1500	1503	✓	✓	✓	✓	✓	✓
78	Pentadecane[b]	1502	1500	×	×	×	✓	✓	✓
79	Decan-5-olide[b]	1508	1514	×	×	×	×	×	×
80	Methyl dodecanoate[b]	1527	1528	×	×	×	×	×	×

Table 3.1 (*continued*)

				1st Set			2nd Set		
No.	**Compound Name**	**RI_{cal} [a]**	**RI_{Lit} [a]**	**GEN**	**MAC**	**SUB**	**GEN**	**MAC**	**SUB**
81	(*E*)-Nerolidol[b]	1573	1572	×	×	×	×	×	×
82	(*Z*)-3-Hexenyl benzoate[b]	1584	1588	✓	✓	✓	×	×	×
83	Hexyl benzoate[b]	1590	1597	✓	✓	✓	×	×	×
84	Isopropyl myristate[b]	1827	1812	✓	✓	✓	×	×	×
85	Hexahydrofarnesylacetone[b]	1850	1838	✓	✓	✓	×	×	×

✓ - identified compounds.

[a] – RI_{cal}- experimental retention index; RI_{Lit} - retention index from literature.

[b] – compounds identified based on comparison with authentic standards and retention index data.

[c] – compounds tentatively identified based mass spectral and retention index data.

[d] – retention index obtained from reference [17].

3.3.4. Comparison of volatile profiles between different *Cyclopia* spp. using multivariate data analysis and correlation with sensory data

Data analysis is often the bottleneck for non-targeted chromatographic analyses due to the size and complexity of the data, and this is especially true for GC×GC. Most often feature extraction such as peak integration using commercial software and subsequent multivariate modelling is used. Shifts in chromatographic data are highly dependent on the chromatographic system and the peripheral equipment used. Hence, the more accurate and reproducible a system is, the more suitable it is for non-targeted GC×GC analysis. It has been shown that the modulator used in this study delivers highly reproducible results, a consequence of the fact that no cooling agent is used. Retention time shifts in both first and second dimensions were very low (**Table 3.2**), which is beneficial since no alignment of the data was necessary for statistical analysis. Prior to two dimensional peak picking, only baseline correction was performed. Automated peak integration was done using an in-house written MATLAB script which defines the beginning and end of each peak by evaluation of the relative change of the slope. Peak areas of all two dimensional slices of a peak were summed to obtain the total peak area. The peak table was then pareto scaled and mean centred. PCA utilising the peak tables was used to investigate differences in volatile composition between the three *Cyclopia* spp. for each harvest year. The scores plots for the GC×GC-FID and sensory data for each of the two sets of honeybush tea samples are shown in **Figure 3.7**.

Figure 3.7A shows the scores plot for GC×GC-FID data of the 2010 honeybush samples, where significant differences are observed between the three species. The first principle component (PC 1) mainly accounts for the differences between the three honeybush species *C. maculata* (MAC), *C. subternata* (SUB) and *C. genistoides* (GEN), and the scores are accordingly clustered horizontally into three groups. Differences within the extracts of each species are mainly explained by PC 2. Clear differentiation of *C. maculata* extracts is also observed based on the sensory data for the same set of samples [20], although *C. subternata* and *C. genistoides* extracts are not as clearly distinguished as observed for the PCA based on GC×GC-FID data (**Figure 3.7C**). *Cyclopia maculata* samples of the 2010 harvest were distinguished by their strong association with the cassia/cinnamon sensory

attribute, as summarised on the aroma spider plot presented in Supplementary Information, **Figure S3.5A**. Identification of the compounds responsible for this differentiation of *C. maculata* according to sensory properties was not possible, since none of the identified compounds responsible for the corresponding segregation based on GC×GC-FID data are associated with the cassia/cinnamon attribute (data not shown). It would therefore be interesting to further investigate the unidentified compounds responsible for the differentiation of the GC×GC volatile profiles by means of time-of-flight (TOF) MS to possibly establish a link with the cassia/cinnamon character of the *C. maculata* samples.

Table 3.2: Summary of the repeatability of retention in both dimensions for the GC×GC separation of selected honeybush tea (*C. maculata*) volatiles using the optimised conditions (**Section 3.2.5.2**). Data were acquired on a single modulator.

		First dimension repeatability			Second dimension repeatability			
[a]Peak No.	Compounds	[b]t_{R1} (min)	Intra-day % RSD[c] (*n*=6)	Inter-day % RSD[c] (*n*=6)	[b]t_{R2} (s)	intra-day % RSD[c] (*n*=6)	Inter-day % RSD[c] (*n*=6)	[d]$W_{1/2}$ height (s)
1	1-pentanol	9.065	0.01	0.00	3.790	1.19	1.00	0.18
9	γ-butyrolactone	16.631	0.00	0.85	1.510	1.62	4.69	0.06
27	2, 6, 6-trimethyl cyclohexen-2-one	24.620	0.00	0.00	2.040	0.00	1.21	0.12
37	(E)-3-nonen-2-one	28.525	0.12	0.12	2.210	1.11	1.11	0.18
56	geranial	35.714	0.00	0.00	2.700	0.00	1.80	0.24
59	undecanal	37.448	0.00	0.00	1.740	0.00	1.93	0.12
73	(E)-β-damascone	42.871	0.00	0.00	2.080	1.49	1.59	0.18
77	(E)-β-ionone	46.042	0.00	0.00	2.360	1.31	1.30	0.18

[a]- peak numbers correspond with **Table 3.1**

[b]- retention time t_{Rx} in the xth dimension

[c]- relative standard deviation

[d]- ^{2}D peak width at half height

n-number of analyses

Distinct differences in the GC×GC volatile profiles of the same *Cyclopia* spp. was not observed for the honeybush samples of 2013 (**Figure 3.7B**). *Cyclopia genistoides* samples are to some extent grouped with low PC1 and PC2 scores, but for the rest no clear differentiation was observed. Sensory analysis of the same samples however, showed clear clustering of the samples into three groups according to species (**Figure 3.7D**). It would therefore seem that the compounds responsible for this sensory differentiation were not included in the PCA of the GC×GC-FID data, either due to the low levels or co-elution with other compounds excluded from the PCA analysis.

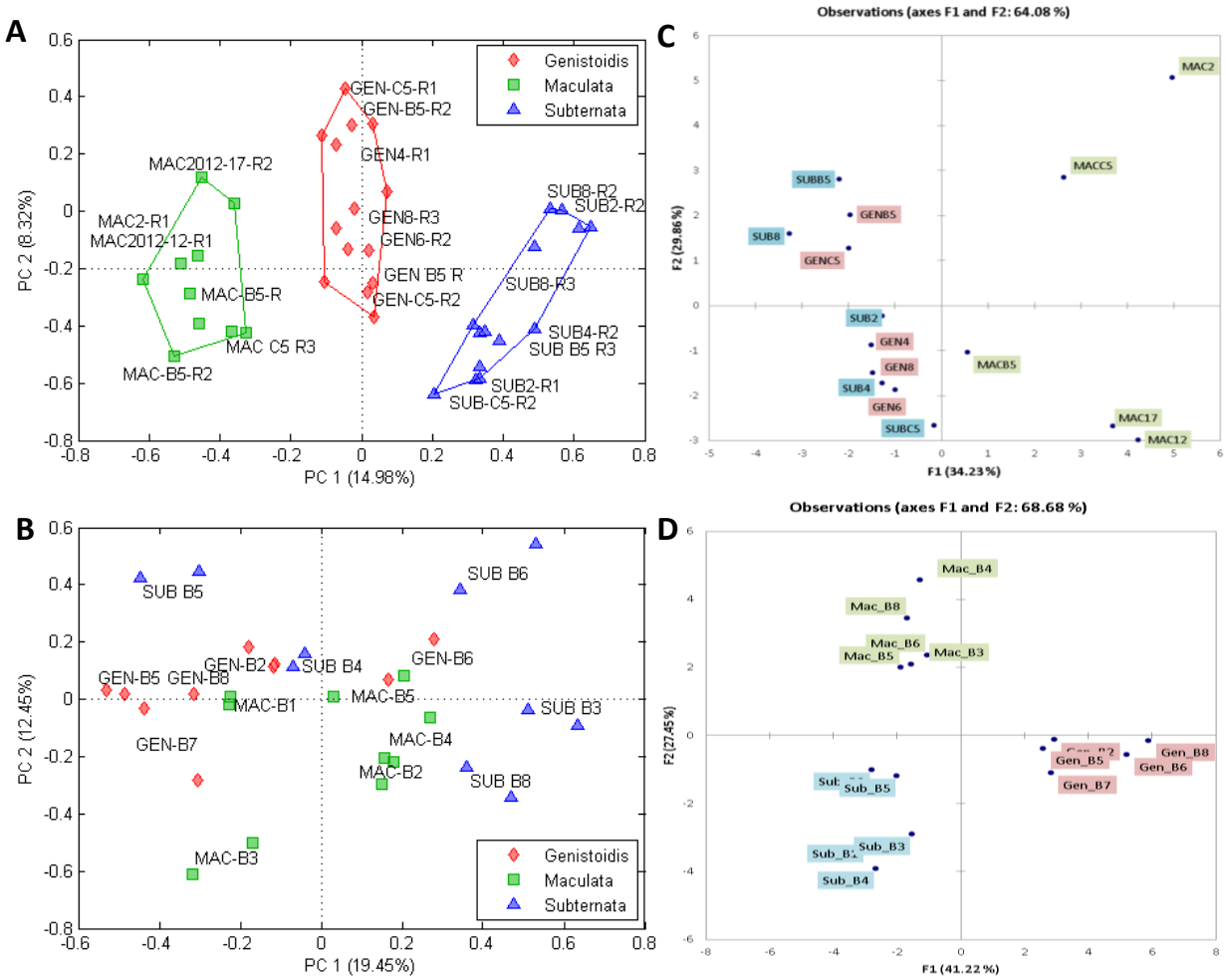

Figure 3.7: Results for the PCA analysis of GC×GC-FID data (**A** and **B**) and sensory data (**C** and **D**) of honeybush tea sample of 2010 (**A** and **C**, set 1) and 2013 (**B** and **D**, set 2) harvest seasons. Plot labels: MAC = *C. maculata*, SUB = *C. subternata*, GEN = *C. genistoides*, R1 and R2 refer to repeat analyses of the same sample by GC×GC-FID.

3.4. Conclusions

A new consumable-free single-stage thermal modulator has successfully been applied for the GC×GC separation of honeybush tea (*Cyclopia* spp.) volatile constituents. Following method optimisation in terms of HS-SPME extraction, the column combination, flow rates, temperature program, discharge voltage and modulation period, good separation of honeybush volatiles was obtained. The modulator provides cost-effective operation and showed excellent performance in terms of retention reproducibility, which proved beneficial in terms of multivariate analysis of the data. Limitations were observed for compounds present at high levels, where breakthrough due to trap overloading was observed. This is however a common problem in the analysis of natural products and beverages due to the wide dynamic range of compound levels in such samples.

Seventy-one compounds were positively identified in three *Cyclopia* spp. using the developed method. Due in part to the limitations of the quadrupole MS used in this study, only 5-methylfurfural was identified for the first time in honeybush tea. Nevertheless, the method proved suitable for the investigation of differences in volatile profiles between the

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three *Cyclopia* spp. Owing to the good performance of the modulator, no pre-alignment of the data was needed for the statistical analysis. PCA of the GC×GC-FID data for the 2010 data set highlighted the unique volatile composition of *C. maculata*, in accordance with sensory analysis of the same set of samples, although the compound(s) responsible for the sensory differentiation could not be identified. This work has illustrated the utility of GC×GC separation for honeybush tea analysis; future work will focus on hyphenation to TOF-MS to allow identification of additional compounds in these samples.

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Supporting Information

Analysis of honeybush tea (*Cyclopia* spp.) volatiles by comprehensive two-dimensional gas chromatography using a novel single-stage thermal modulator

Supporting Information

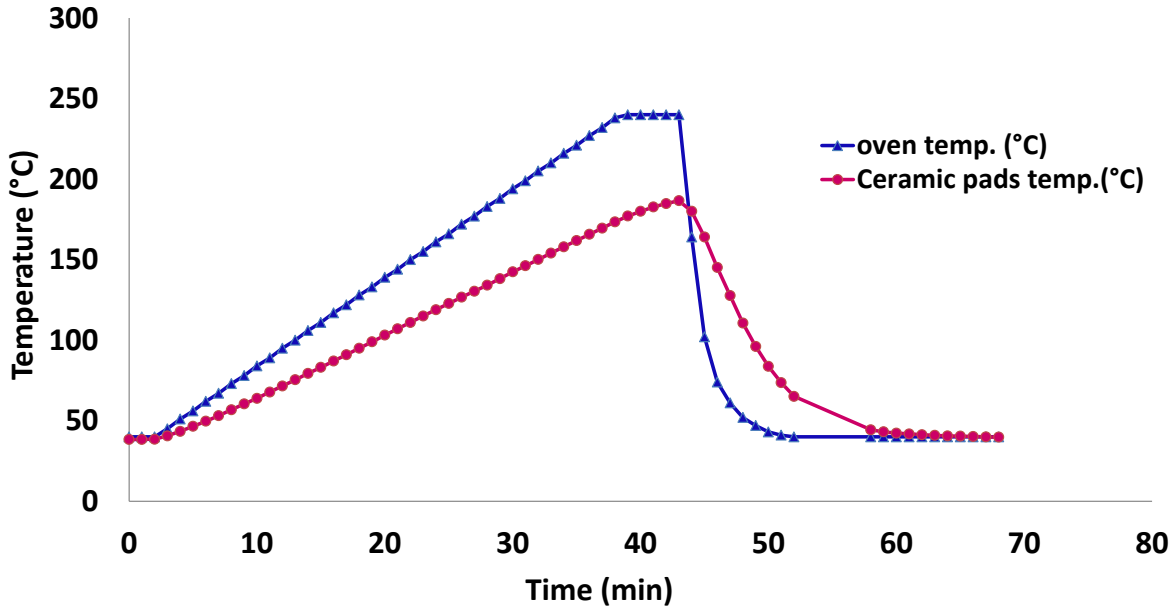

Figure S3.1: Relative temperature difference between the modulator ceramic plates (as measured using a thermocouple placed between them) and the oven temperature (according to the GC), measured using an oven temperature ramping rate of 5.5°C/min from 40°C to 240°C.

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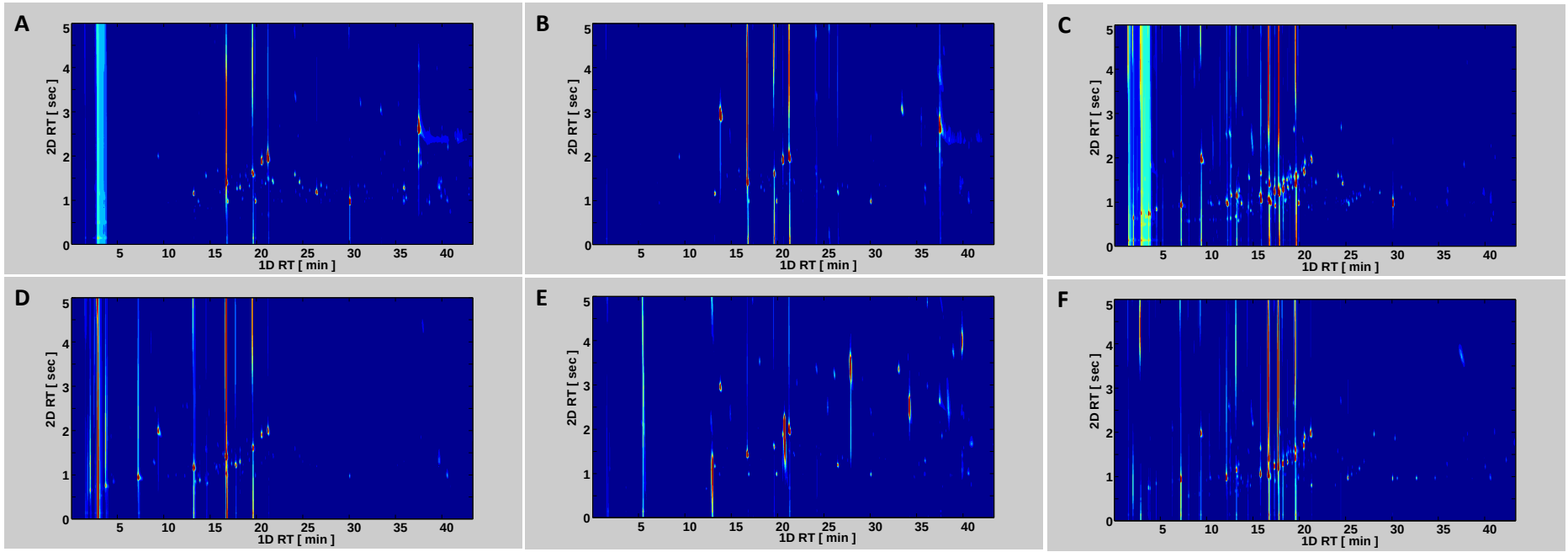

Figure S3.2: GC×GC-FID contour plots of honeybush tea volatiles obtained for different SPME fibres (**A)** PDMS, (**B)** PA, (**C**) DVB/CAR/PDMS, (**D**) CAR/PDMS, (**E**) PEG and (**F**) PDMS/DVB. The analyses were carried out on a HP-5MS × Stabilwax column combination at an inlet headpressure of 151.7 kPa; F = 2.05 mL/min; oven temperature ramping rate = 5.5 °C/min (refer to **section 3.2.5.1** for further experimental details).

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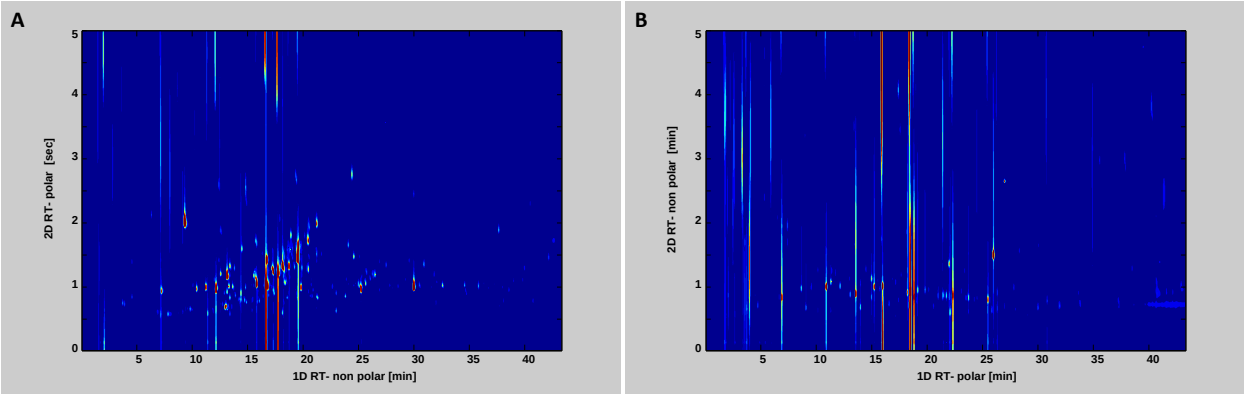

Figure S3.3: GC×GC-FID contour plots obtained for the analysis of honeybush tea volatile components on different column sets: (**A**) non-polar × polar (HP-5MS × Stabilwax) and (**B**) polar × non-polar (DB-WAXERT× Rxi-5Sil MS) column combinations. The analytes were extracted by HS-SPME using a PDMS/DVB SPME fibre and chromatographic conditions are as specified in **Figure S3.2**.

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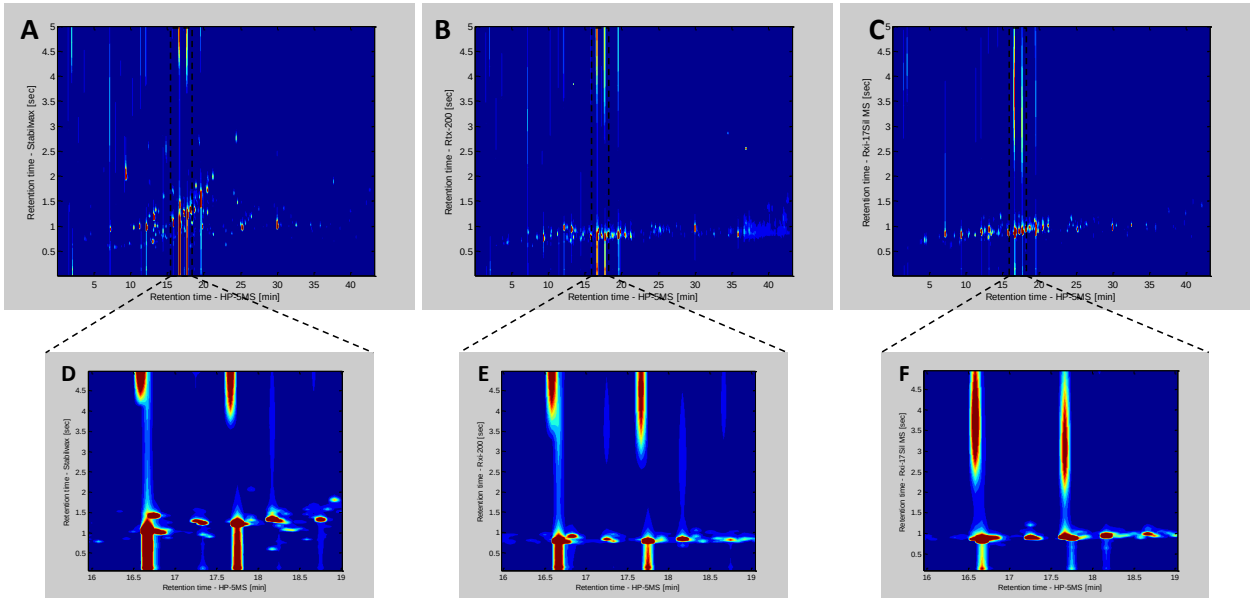

Figure S3.4: Influence of polarity of the second dimension column on wraparound in the GC×GC separation of honeybush tea volatile components. A HP-5 MS column was used in ^{1}D, together with (**A**) Stabilwax, (**B**) Rtx-200 and (**C**) Rxi-17Sil MS columns in ^{2}D. Zoomed contour plots show the degree of wraparound for two major compounds on the (**D**) Stabilwax, (**E**) Rtx-200 and (**F**) Rxi-17Sil MS ^{2}D columns. A PDMS/DVB SPME fibre was used for extraction of analytes and chromatographic conditions are the same as in **Figure S3.2**.

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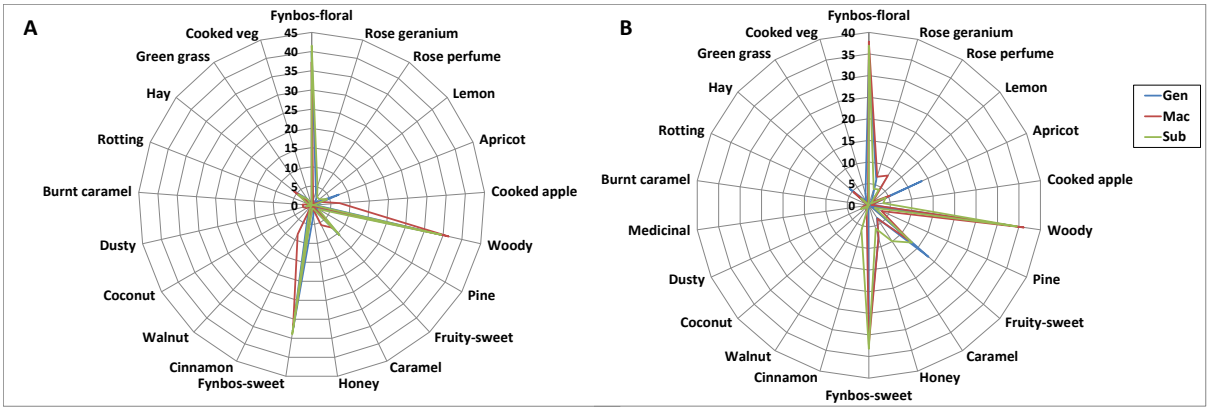

Figure S3.5: Spider plots showing the mean scores of aroma profiles for *Cyclopia* species [*C. genistoides* (Gen), *C. maculata* (Mac) and *C. subternata* (Sub)], (**A**) sample set 1 and (**B**) sample set 2. Data were collected as specified in **Section 3.2.7.**

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Chapter 4

Detailed qualitative analysis of volatile compounds of honeybush tea (*Cyclopia* spp.) by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOF-MS)*

**This chapter is in preparation for publication*

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Detailed qualitative analysis of volatile compounds of honeybush tea (*Cyclopia* spp.) by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOF-MS)

Abstract

The volatile composition of honeybush (*Cyclopia*) species was studied by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOF-MS). Headspace-solid phase micro-extraction (HS-SPME) was used to extract the volatile compounds from tea infusions of *C. genistoides*, *C. maculata* and *C. subternata*. A total of 272 compounds were identified; 73 of these were identified with reference standards and the rest were tentatively identified using mass spectral and retention index (RI) data. The identification power of TOF-MS enabled the tentative identification of 158 new volatile compounds in honeybush tea. The majority of the compounds identified were common to all three *Cyclopia* species, although there were differences in their relative abundances, and some compounds were unique to each of the species. In *C. genistoides*, *C. maculata* and C*. subternata* 232, 217, 181 compounds were identified, respectively. This study further emphasises the complexity of honeybush tea's volatile composition and confirms the power of GC×GC combined with TOF-MS for the analysis of such complex samples.

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4.1. Introduction

Honeybush tea, prepared from the plant material of the genus *Cyclopia* Vent. species (family Fabaceae, tribe Podalyrieae) is considered South Africa's sweetest herbal tea [1]. Honeybush shrubs are endemic to the coastal plains and mountain regions of the Western and Eastern Cape provinces. Most of the honeybush tea product available on the market constitutes more than one *Cyclopia* species. *Cyclopia genistoides*, *C*. *intermedia*, and *C*. *subternata* are currently mostly used for commercial production. However, as the industry is rapidly expanding, the species *C*. *maculata*, *C*. *sessiliflora* and *C*. *longifolia* are also being investigated for commercial production of honeybush tea [1]. It is known that blending of species can have an important effect on the sensory properties of the final product. It is therefore important to characterise each of the individual species before blending in order to preserve the unique 'honeybush tea' flavour. Until recently, there have been no formal terminologies to describe the characteristic honeybush tea aroma, nor has a method to determine the differences between *Cyclopia* species been established. This has resulted in teas of different qualities being sold on the market. The quality of tea is typically determined by sensory properties such appearance, aroma and taste. A honeybush 'sensory wheel', similar to that previously reported for rooibos tea [2], has recently been developed for use in sensory studies and as a quality control tool [3].

Aroma is one of the most important properties used for assessment of the quality of tea, and correlation of the volatile chemical compounds responsible for the aroma characteristics is an equally important step in the quality control and manufacturing of tea. To this end, gas chromatography hyphenated to mass spectrometry (GC-MS) and olfactometry (GC-O) have previously been used for the analysis of the volatile fraction of honeybush tea, and more than 200 compounds were identified. These belong to a range of different classes, with terpenoids constituting the highest percentage of compounds, followed by aldehydes, esters, ketones, hydrocarbons, alcohols, furans and others [4–6]. Notwithstanding the power of one dimensional (1D) capillary gas chromatography, it is not possible to completely resolve all the volatile components of complex natural products such as honeybush tea, thus preventing complete identification of all constituents.

Comprehensive two-dimensional gas chromatography (GC×GC) is increasingly being used as an alternative, since this technique offers much higher resolving power than 1D GC. This is a result of sequential separation of analytes on two different GC columns [7,8]. A typical example is a non-polar × polar column set, where analytes are first separated according to their boiling point on the non-polar column and subsequently separated according to their polarity on the polar column. The two columns are connected by a modulator, whose function is to trap effluent from the first dimension (^{1}D) column, focus analytes into narrow bands and subsequently re-inject them onto the second dimension (^{2}D) column. Cryogenic modulators such as used in this study are most common, and typically produce narrow peaks (in the order of 100 ms) in the second dimension. In order to obtain sufficient data points across such narrow peaks for accurate identification, a very fast detector response time is required [9,10]. Since the first generation GC×GC instruments, flame ionization detection (FID) has been the detector of choice due to its fast duty cycle (up to 500 Hz). However, the FID is mainly applicable in demonstrating the high resolution of this technique and for quantitative GC×GC analysis, and does not allow identification of analytes. Identification using FID may be achieved by

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comparison of retention times with reference compounds, but this approach is not feasible when the sample has never been characterised before. Mass spectrometry hyphenated to GC has been used for many years for the identification of components separated by GC. In chapter 3, quadrupole mass spectrometry (qMS) was used in combination with GC×GC separation. However, due to the slow duty cycle of this analyser, the scan range was reduced to achieve the maximum acquisition rate of 22 Hz. Because of this, identification of the majority of (non-standard) compounds in honeybush tea was not possible. Thus far, time-of-flight mass spectrometry (TOF-MS) has proven to be the best MS detector for use in combination with GC×GC separation, since this detector is capable of providing the fast acquisition rates (up to 500 Hz) required for accurate identification of components separated by GC×GC [9]. Even when incomplete separation is obtained, TOF-MS offers an additional means of separation based on deconvolution of MS data [11]. Therefore TOF-MS was used in this study to enable identification of new honeybush tea volatile constituents.

GC, like most analytical techniques, cannot handle all sample matrices directly and since tea volatiles typically occur at low concentrations, isolation and pre-concentration of the volatile compounds is required prior the GC analysis. Sorptive sample preparation techniques have taken prevalence over distillation and solvent extraction methods as they minimize sample loss and extraction time and are environmentally friendly. For example, the sample enrichment probe (SEP) was previously used in combination with GC-MS for the analysis of honeybush tea volatiles [12]. The hardware used in SEP consists of a thin rod of an inert material coated with a short sleeve of polydimethylsiloxane (PDMS) rubber at one end. This method provides high sample enrichment, as is evident from its application to honeybush tea. However, SEP requires a more elaborate injection procedure, which makes the method practically challenging in most laboratories. Solid phase micro-extraction (SPME), though less sensitive than SEP, has extensively been used for analysis of a vast range of food samples [13,14]. It is simple to use and can be automated without the need to alter GC instrumentation.

In light of the above, the aim of the work reported in this chapter was to use GC×GC-TOF-MS for the detailed qualitative analysis of the volatile constituents of honeybush tea. GC×GC was used in combination with headspace-solid phase micro-extraction (HS-SPME) for the analysis of three *Cyclopia* species, namely *C. genistoides, C. maculata*, and *C. subternata*. The volatile composition of these three species was elucidated and compared, with special emphasis on attempting to identify compounds responsible for the divergent aroma properties of these species.

4.2. Material and methods

4.2.1. Chemicals and materials

A standard mixture consisting of 108 volatile organic compounds was kindly supplied by Laboratory of Ecological Chemistry (LECUS, Stellenbosch University, SA). Standards were synthesised where commercial products were not available [6], and the rest were purchased from Sigma-Aldrich or Fluka (St. Louis, MO, USA). SPME was performed using a 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibre purchased from Supelco (Bellefonte,

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PA, USA). The C_6-C_{40} linear alkane mixture and sodium chloride were obtained from Sigma-Aldrich and AAA-Chemicals (La Marque, TX, US), respectively. Dichloromethane (DCM) used to dilute the linear alkanes mixture was also purchased from Sigma-Aldrich.

4.2.2. Tea samples

A total of 15 honeybush tea infusions (consisting of 5 samples each of the *Cyclopia* species *C. genistoides*, *C. maculata* and *C. subternata*) were provided by the Department of Food Science (Stellenbosch University, South Africa). The plant material was sourced from commercial processors or were prepared by laboratory-scale processing at the Post-Harvest & Wine Technology Division of ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa) during the 2010 and 2011 harvesting seasons [3]. The tea was prepared as described in section **3.2.2**; the infusions were prepared by adding 900 g boiled deionised water to 11.25 g of tea leaves. Tea leaves were infused for 5 min, strained into glass bottles and cooled to room temperature before being frozen (-4°C) until analysis.

4.2.3. Headspace-solid phase micro-extraction (HS-SPME) procedure

The two-phase PDMS/DVB SPME fibre was used for the extraction of honeybush tea volatiles. The fibre was conditioned according to the specifications of the manufacturer prior to use. Tea infusions were defrosted at room temperature, and 10 mL of tea infusion was placed in 20 mL vial containing 2 g NaCl. The tea was pre-incubated at 30°C for 3 min while stirring with a magnetic stir bar at 500 rpm. The SPME fibre was then exposed to the headspace of the tea (30°C) for 30 min at an agitation speed of 100 rpm; subsequently the analytes were desorbed in the GC injector at 240°C for 10 min. All analyses were performed in duplicate.

4.2.4. GC×GC conditions

Analyses were carried out on LECO instrument (LECO Corp., St. Joseph, MI, USA) consisting of an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) equipped with a split/splitless injector, Gerstel MPS (multi-purpose sampler) autosampler (Mulheim ad Ruhr, Germany) and a dual stage cryogenic modulator (LECO) coupled to a Pegasus IV TOF-MS detector (LECO). Helium was used as a carrier gas at a constant flow of 1.14 mL/min. The injector was operated at 240°C in split mode (1:10 split ratio) for liquid injections and in splitless mode for SPME injections (splitless for 2 min). The solvent delay for liquid injections was 5 min. The column set consisted of a non-polar 30 m × 0.25 mm i.d. (internal diameter) × 0.25 µm d_f (film thickness) Rxi-5Sil MS (Restek, Penn Eagle Park, CA, USA) column in the first dimension (^{1}D) and a polar 0.8 m × 0.25 mm ID × 0.25 µm d_f Stabilwax (Restek) column in the second dimension (^{2}D). A modulation period of 5 s was used for all analyses with the cryogenic trap cooled to -196°C using liquid nitrogen. The temperature of the GC oven was programmed from 40°C (2 min hold time) to 240°C (5 min hold time) at 5°C/min. The secondary oven offset temperature was +10°C relative to the GC oven. The transfer line and ion source were set to 250°C and 200°C, respectively, and the detector voltage was 1650 V. Data were acquired at a rate of 100 spectra/sec with mass scan range of 45-400 amu.

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4.2.5. Data processing method

Data processing was performed using ChromaTOF®-GC software (LECO Corp., version 4.50.8.0) incorporating an algorithm for peak deconvolution. The ^{1}D and ^{2}D peak widths were set to 25 s and 0.4 s, respectively. The percentage match required to combine the modulated peaks was set to 65%, with a minimum signal-to-noise (S/N) of 50 for all sub-peaks. Identification was performed using reference standards (108 compounds), and where not available tentative identification was by MS library search using the NIST 11 library and comparison of calculated ^{1}D linear retention indices (LRI_{cal}) with literature values (LRI_{Lit}). The minimum similarity match factor for spectral matching was set to 70%.

4.3. Results and discussion

4.3.1. Separation of honeybush tea volatiles

Volatile compounds of honeybush tea were extracted by SPME using a PDMS/DVB fibre. SPME offers the advantage of isolation and pre-concentration in one single uninterrupted step, thus shortening the extraction time and minimising the loss of volatile compounds [15]. An autosampler capable of automated SPME injection was used in this study, which substantially reduced first dimension retention time shifts compared to manual injection used in chapter 3. The average percentage relative standard deviation (%RSD) values were 0.02 and 0.43 % for the first and second dimensions, respectively (**Table 4.1**). This corresponds to ±1 modulation period in the first dimension, and is lower than observed for the single-stage thermal modulator with manual injection. The retention time shift in the ^{2}D was mainly due to the timing of the modulator [16]. Nonetheless, shifts in the positions of the compounds on the two-dimensional chromatographic space were insignificant, which simplifies the identification process as one sample could be used to identify the common compounds found in the other samples.

Table 4.1: Reproducibility data for the retention times of selected honeybush tea volatile compounds determined on the commercial GC×GC-TOF-MS instrument.

Compound	1st dimension		2nd dimension	
	t_R[a] (sec)	%RSD[b] (*n=6*)	t_R[a] (sec)	%RSD[b] (*n=6*)
Pentanal	185	0.00	0.99	0.76
2,2,6-Trimethyl-cyclohexanone	715	0.00	1.26	0.41
(*E*)-3-Nonen-2-one	905	0.00	1.57	0.35
Undecanal	1195	0.00	1.18	0.64
β-Damascone	1355	0.00	1.53	0.34
(*E*)-β-Ionone	1461	0.14	1.69	0.24
Benzophenone	1675	0.00	4.05	0.24

[a] retention time; [b] relative standard deviation; *n*- number of analyses

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Separation was performed on a non-polar × polar column set, where compounds are first separated based on differences in their vapour pressures in the ^{1}D column (Rxi-5Sil MS) and then according to selective interactions in the ^{2}D dimension (Stabilwax) column. While it is common practice to use a narrow bore column in the second dimension to achieve better performance for very fast separations, this typically results in non-optimal carrier gas velocities (below and above the optimum values in the ^{1}D and ^{2}D, respectively) and may also result in overloading of the ^{2}D column [10,17]. In this work, a second dimension column with the same diameter and film thickness (0.25 mm, 0.25 µm) as the first dimension column was used to minimise overloading of the second dimension column [16], which is especially a concern in natural product analysis, where compound concentrations may span several orders of magnitude.

Figure 4.1 depicts a portion of the contour plot obtained for the analysis of a honeybush tea sample, and illustrates the separation power of GC×GC: the *cis*-2,6-dimethyl-2,6-octadiene (**36**) and *trans*-2,6-dimethyl-2,-6-octadiene (**46**) isomers have the same polarity and co-elute on the wax column, but are separated on the apolar column, whilst for myrcene (**39**) and (*E*,*E*)-2,8-decadiene (**43**) the opposite occurs - these compounds co-elute in ^{1}D but are separated in the ^{2}D column. The improved separation of other isomers like α/γ-terpinene (**55, 71**) and (*Z*)/(*E*)-β-ocimene (**61, 66**) is also evident (**Figure 4.1**).

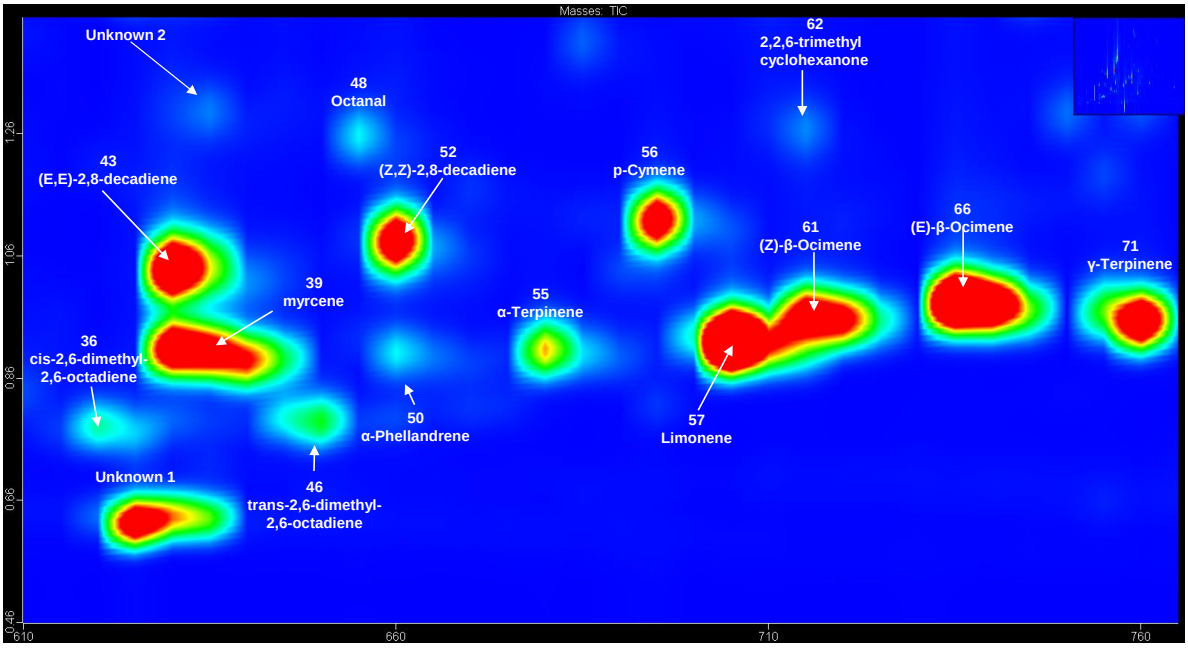

Figure 4.1: Section of the total ion chromatogram (TIC) GC×GC contour plot illustrating the separation power of GC×GC separation for some volatile compounds of *C. genistoides*. Peak numbers correspond to **Table 4.2**.

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Notwithstanding the resolving power of GC×GC, the relatively fast oven ramping rate used in this study resulted in partial co-elution of some compounds. On such cases, the fast scanning rates of the TOF-MS offers the advantage of deconvolution to enable resolving partially co-eluting peaks [11,18]. **Figure 4.2** demonstrates the power of deconvolution: the partially co-eluting compounds (*E*)-methyl-3,5-heptadien-2-one (**92**) and hotrienol (**93**) can be selectively detected using extracted ion data for m/z 109 and 71, respectively, obtained as the unique ions for these compounds by mass spectral deconvolution. It can also be seen that the highly abundant linalool (**90**) partially overlaps with both (*E*)-6-methyl-3,5-heptadien-2-one and hotrienol in the first dimension, but is completely separated from both in the second dimension using the relevant extract ion data. Indeed, this is an example of one of the challenges encountered in 1D GC analysis of natural products, as it is often impossible to accurately identify and quantify closely eluting compound varying widely in concentration.

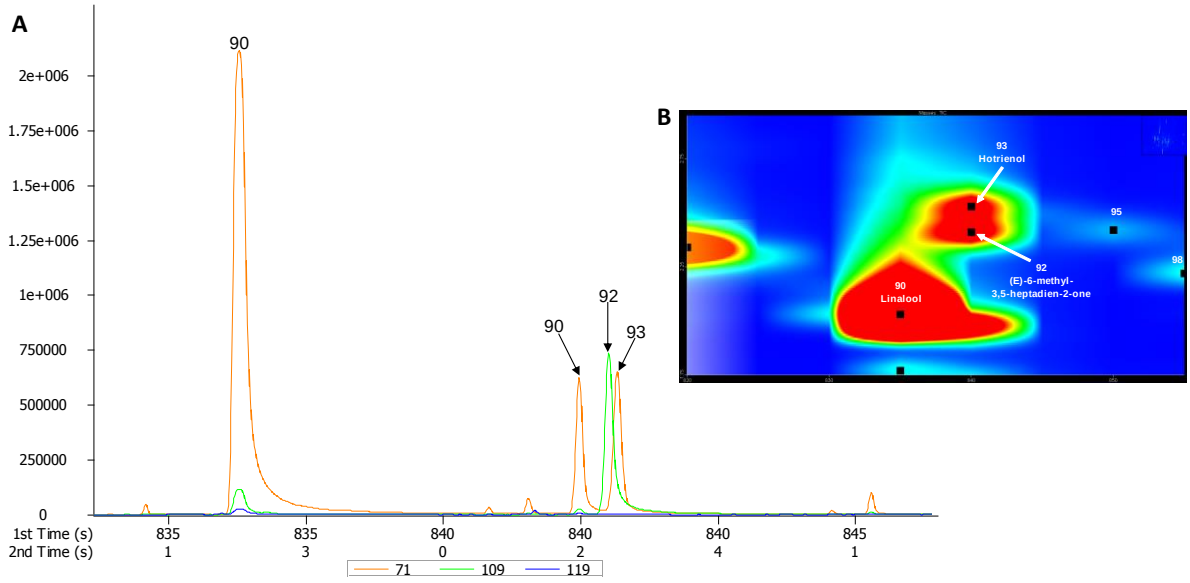

Figure 4.2: (**A**) GC×GC-TOF-MS extracted ion chromatogram based on deconvoluted mass spectra, plotted for m/z 71, 109 and 119. Compounds linalool (**90**), (*E*)-6-methyl-3,5-heptadien-2-one (**92**) and hotrienol (**93**) are indicated. (**B**) shows the corresponding part of the total ion chromatogram (TIC) contour plot. Peak numbers correspond to **Table 4.2**.

4.3.2. Identification of honeybush tea volatiles

Compounds were identified based on comparison of retention times and MS spectra with 108 authentic standards, and tentative identification was performed by comparing MS spectra with the NIST 11 library and retention indices (RI) obtained from NIST and PHEROBASE databases. The minimum MS match factor was set to 700 (out of 1000) and only differences of less than 25 units between calculated (RI_{Cal}) and literature (RI_{Lit}) retention indices were allowed.

Data analysis was performed using the automated instrument software (ChromaTOF). Experimental retention index data were generated by injection of linear hydrocarbons, and the ^{1}D dimension retention times were incorporated into the software to automatically calculate

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retention indices of the sample components. More than a thousand chromatographic peaks were generated when an automated data processing method was used. To maximise identification certainty, the number of peaks processed was limited to 1000 with a minimum S/N of 50; further processing parameters are outlined in section **4.2.5**.

Identification was primarily based on the comparison of retention times and mass spectral data with 108 authentic standards. Based on the high MS match factors obtained, good separation of the standards (an example of the contour plot obtained for the standards is shown in **Figure 4.3**) and good reproducibility of the data set (see also **Table 4.1**), seventy three compounds were easily identified as detailed in **Table 4.2**. The remaining 36 standards were not found in the honeybush tea species analysed here. An additional 199 compounds were tentatively identified using MS and retention index data. In total 272 compounds were therefore identified in the three *Cyclopia* species analysed, which comprised of terpenoids, alkanes, alcohols, ketones, acids, furan and pyrans (**Table 4.2**).

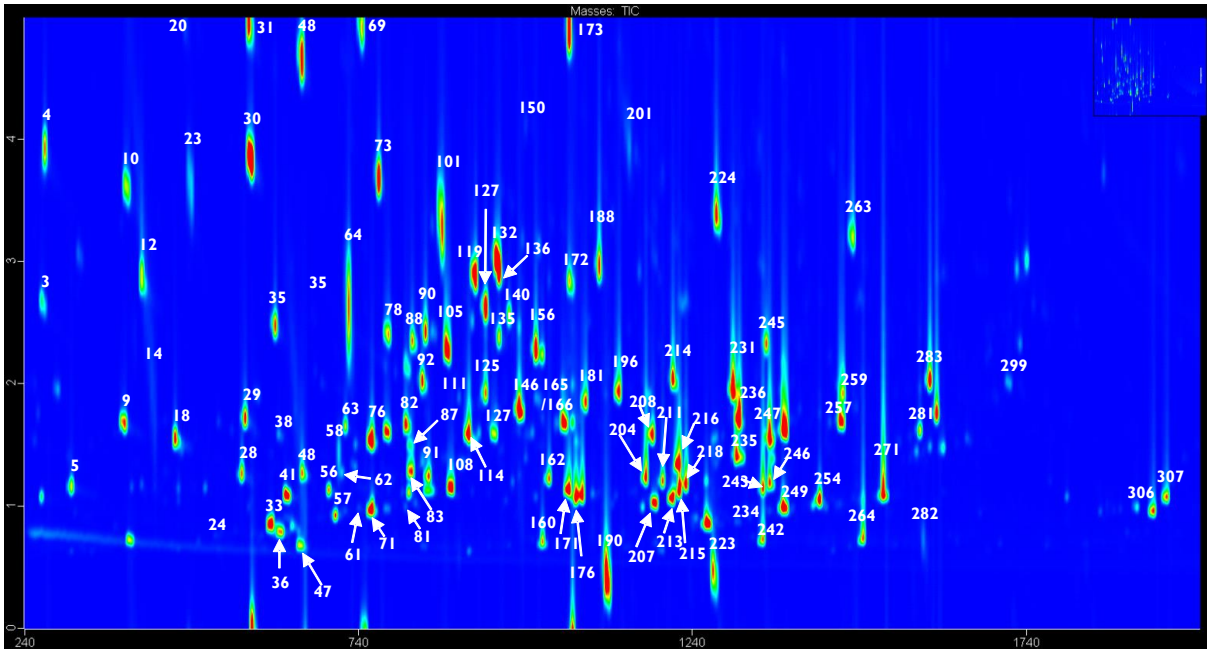

Figure 4.3: Contour plot obtained for the GC×GC-TOF-MS analysis of honeybush tea reference standards. Peak numbers correspond to **Table 4.2**.

As previously reported, the majority of the volatile compounds of honeybush tea are terpenoids. Identification of terpenoids in the absence of reference standards is however challenging because of the similarity of many of their mass spectra; retention index data were therefore particularly important [19]. One example of such a case is the identification of compound **39**, shown in **Figure 4.4**. A library search gave β-pinene as the most probable identity, with a similarity of 900, and myrcene as a second possibility (similarity 800). However, β-pinene and myrcene have virtually indistinguishable mass spectra, as illustrated in **Figure 4B** and **Figure 4C**. Using the retention index data for the unknown (RI=989), and literature values of β-pinene (RI=979) and myrcene (RI=991), compound **39** was tentatively assigned as myrcene [20].

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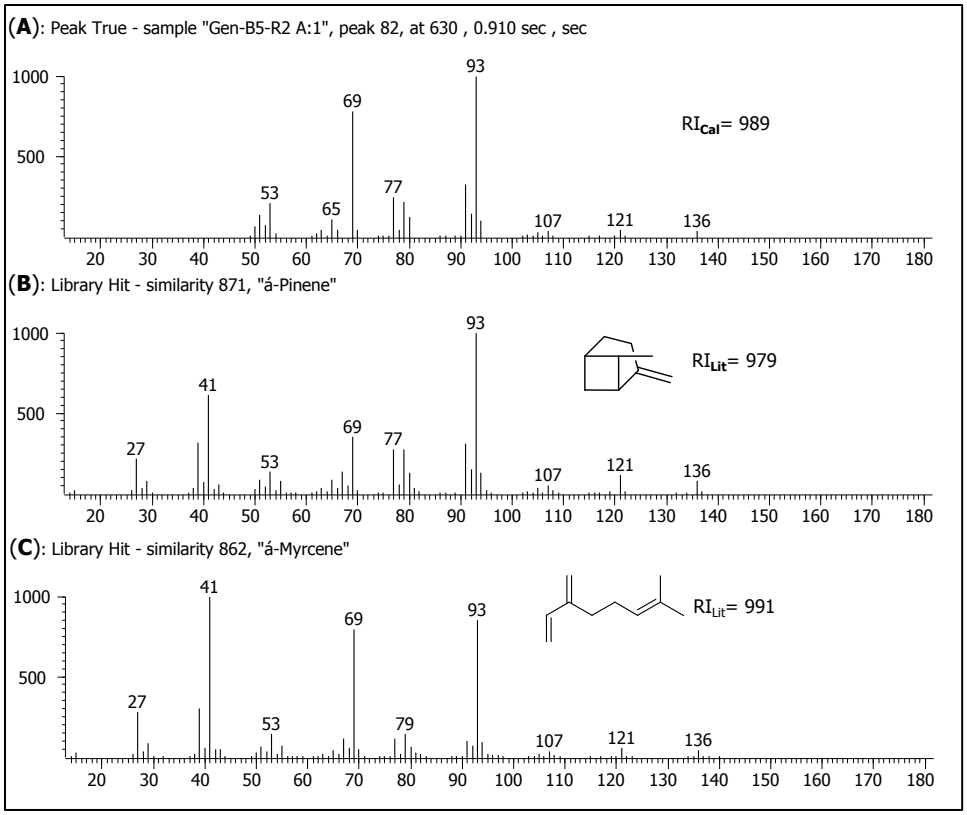

Figure 4.4: Illustration of tentative identification of α-myrcene (compound **39** in **Table 4.2**) using mass spectral and retention index data; (**A**) MS spectrum of unknown peak in *C.genistoides*, (**B**) α-pinene MS spectrum and (**C**) α-myrcene MS spectrum.

232, 217 and 181 compounds were identified in *C. genistoides*, *C. maculata* and *C. subternata* samples, respectively. Example contour plots for each of these species are presented in **Figure 4.5**. Differences were noted in the volatile compositions of each of the species, both in terms of relative concentrations and the identified compounds. For example, as can be seen in **Figure 4.5**, eugenol (**223**) was observed at relatively high concentrations in *C. genistoides* and *C. maculata*, whereas in *C. subternata* much lower levels were observed. Similarly, α-terpineol (**156**) levels were high in *C. genistoides* and *C. subternata* and low in *C. maculata*. Furthermore, a few compounds were uniquely detected in each species (**Table 4.2**). For example, citral (**180**) and phenylethyl alcohol (**230**) were only detected in *C. genistoides*, 3-heptanone (**16**) and 3,4-dimethyl-2,5-furandione (**60**) in *C. subternata* and 1,8-cineol (**59**) in *C. maculata*. Of particular interest is the compound (*E*)-cinnamaldehyde (**199**), which was only detected in *C. maculata* samples. This compound is a major constituent of cinnamon essential oil, and is associated with cinnamon, sweet and rose-apple flavour descriptors [21]. (*E*)-cinnamaldehyde is therefore most likely responsible for the characteristic cassia/cinnamon aroma ascribed to the same set of *C. maculata* samples by sensory analysis [3]. It is noteworthy that this compound was not detected in the *C. genistoides* and *C. subternata* samples, which further supports the hypothesis that (*E*)-cinnamaldehyde is responsible for the unique sensory profile of the *C. maculata* samples (Chapter 3, **Figure S3.5A**). In total 33, 30 and 2 unique compounds were detected in *C.*

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genistoides, *C. maculata* and *C. subternata*, respectively. It should however be noted that species-specific compounds cannot be confirmed based on the data attained for such a relatively small sample set. It would therefore be interesting to investigate a larger batch of samples to verify these findings.

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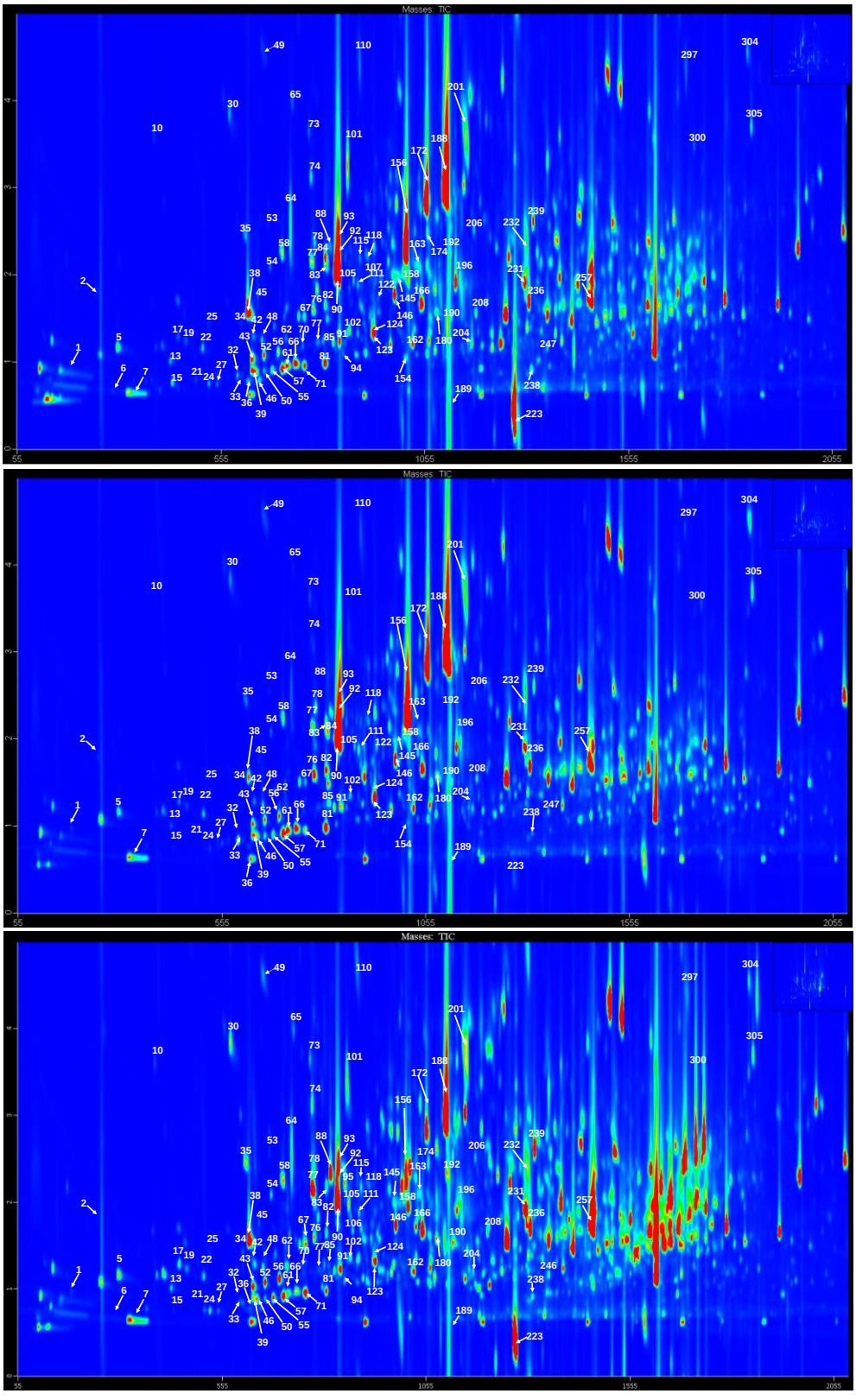

Figure 4.5: TIC GC×GC-TOF-MS contour plots obtained for the analysis of honeybush tea species: (**A**) *C. genistoides*, (**B**) *C. subternata* and (**C**) *C. maculata*.

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Table 4.2: The volatile compounds identified in honeybush tea by GC×GC-TOF-MS.

No.	Compound name	RI_{Cal}[c]	RI_{Lit}[d]	*GEN*[e]	*MAC*[e]	*SUB*[e]	Identification method[f]	Ref.[g]
1	Pentanal	722.5	722	✓	✓	✓	MS,RI	
2	4-Methyl-2-pentanol[a]	763.6	760	✓	✓	✓	MS,RI	
3	1-Pentanol	773.6	771	×	×	×	STD	
4	(*Z*)-2-Penten-1-ol	777.5	774	×	×	×	STD	
5	Hexanal	794.5	793	✓	✓	✓	STD,MS,RI	
6	2-Octene[a,b]	800.4	799	✓	✓	✓	MS,RI	
7	1,3-Octadiene[a,b]	819.2	826	✓	×	×	MS,RI	
8	1,2,5,5-Tetramethyl-1,3-cyclopentadiene[a]	838	840	✓	✓	✓	MS,RI	
9	(*E*)-2-Hexenal	851.1	855	×	×	×	STD	
10	(*Z*)-3-Hexen-1-ol	852.3	853	✓	✓	✓	STD,MS,RI	
11	3-Methyl butanoic acid	859.1	858	×	×	×	STD	
12	1-Hexanol	867.4	871	✓	×	✓	STD,RI,MS	
13	(*E*,*E*)-1,3,6-Octatriene	875.6	880	✓	✓	✓	MS,RI	
14	2-Methyl butanoic acid	876.3	875	×	×	×	STD	
15	2,6-Dimethyl-1,5-heptadiene[a]	878.6	882	✓	✓	✓	MS,RI	
16	3-Heptanone[a]	882	887	×	×	✓	MS,RI	
17	2-Heptanone	885.2	884	✓	✓	✓	MS,RI	
18	(*Z*)-4-Heptenal	897.8	895	✓	✓	✓	STD,MS,RI	
19	Heptanal	900.7	901	✓	✓	✓	MS,RI	
20	2-Acetylfuran	908.3	912	×	✓	×	STD,MS,RI	
21	3-Thujene[a]	922.7	923	✓	✓	✓	MS,RI	
22	Methyl hexanoate	922.9	922	✓	✓	✓	MS,RI	
23	γ-Butyrolactone	923.8	922	✓	✓	✓	STD,MS,RI	
24	α-Pinene	931	939	✓	✓	✓	STD,MS,RI	
25	2,7-Dimethyl-oxepine[a]	931.3	934	✓	✓	×	MS,RI	
26	Citronellene[a]	942.1	943	✓	✓	✓	MS,RI	
27	Camphene	947.7	948	✓	✓	✓	MS,RI	

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Table 4.2: (*continued*)

No.	Compound name	RI_{Cal}[c]	RI_{Lit}[d]	*GEN*[e]	*MAC*[e]	*SUB*[e]	Identification method[f]	Ref.[g]
28	6-Methyl-2-heptanone	953.5	956	✓	✓	✓	STD,MS,RI	
29	(*E*)-2-Heptenal	956.5	957	×	×	×	STD	
30	Benzaldehyde	960.5	960	✓	✓	✓	STD,MS,RI	
31	5-Methyl furfural[a]	961.1	964	×	×	×	STD	
32	2,2,6-Trimethyl-6-vinyltetrahydropyran[b]	969.9	971	✓	✓	✓	MS,RI	
33	β-Pinene	975.5	979	✓	✓	✓	STD,MS,RI	
34	1-Octen-3-one[a]	975.8	975	✓	✓	✓	MS,RI	
35	1-Octen-3-ol	981.9	981	✓	✓	✓	STD,MS,RI	
36	(6*Z*)-2,6-Dimethyl-2,6-octadiene	983.8	990	✓	✓	✓	STD,MS,RI	
37	3-Octanone[a]	984	988.7	✓	✓	✓	MS,RI	
38	6-Methyl-5-hepten-2-one	984.2	985	✓	✓	✓	STD,MS,RI	
39	Myrcene	989.4	991	✓	✓	✓	MS,RI	
40	2,3-Dehydro-1,8-cineole[a]	989.4	990	×	✓	✓	MS,RI	
41	2-Pentylfuran	989.5	993	×	×	×	STD	
42	2-Octanone[a]	989.6	989.4	✓	✓	✓	MS,RI	
43	(*E*,*E*)-2,8-Decadiene	989.46	995	×	✓	×	MS,RI	[22]
44	Hexanoic acid	995.7	995	✓	✓	×	MS,RI	
45	3-Octanol[a]	998.2	998	✓	✓	✓	MS,RI	
46	(6*E*)-2,6-Dimethyl-2,6-octadiene	1000.4	1004	✓	✓	✓	MS,RI	
47	Decane	1000.4	1000	×	✓	×	STD,MS,RI	
48	Octanal	1003.4	1004	✓	✓	✓	STD,MS,RI	
49	2-Formyl-1-methylpyrrole	1005.2	1022	✓	×	×	STD,MS,RI	
50	α-Phellandrene	1005.9	1005	✓	✓	✓	MS,RI	
51	Carbitol[a] [2-(2-ethoxyethoxy)-Ethanol]	1007.9	1006	✓	✓	✓	MS,RI	
52	(*Z*,*Z*)-2,8-Decadiene	1006	1001	✓	×	×	MS,RI	[22]
53	(*E*,*E*)-2,4-Heptadienal	1012.2	1012.3	✓	✓	✓	STD,MS,RI	
54	1,4-Cineol[a]	1014	1015	✓	✓	✓	MS,RI	

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Table 4.2: (*continued*)

No.	Compound name	RI_{Cal}[c]	RI_{Lit}[d]	*GEN*[e]	*MAC*[e]	*SUB*[e]	Identification method[f]	Ref.[g]
55	α-Terpinene	1016.7	1017	✓	✓	✓	MS,RI	
56	p-Cymene	1024.9	1025	✓	✓	✓	STD,MS,RI	
57	Limonene	1030.2	1031	✓	✓	✓	STD,MS,RI	
58	2-Ethyl-1-hexanol[a]	1030.9	1030	✓	✓	✓	MS,RI	
59	1,8-Cineol[a]	1032.9	1031	×	✓	×	MS,RI	
60	3,4-Dimethyl-2,5-furandione	1033.2	1038	×	×	✓	MS,RI	
61	(*Z*)-β-Ocimene	1035.7	1038	✓	✓	✓	STD,MS,RI	
62	2,2,6-Trimethyl-cyclohexanone	1035.8	1035.8	✓	✓	✓	STD,MS,RI	
63	(*E*)-3-Octen-2-one	1038.7	1040	✓	✓	✓	STD,MS,RI	
64	Benzyl alcohol	1044.5	1041	✓	✓	✓	STD,MS,RI	
65	Benzeneacetaldehyde[a]	1045.4	1045	✓	✓	✓	MS,RI	
66	(*Z*)-β-Ocimene	1046.5	1044	✓	✓	✓	MS,RI	
67	2-methyl-6-methylene-2-Octene	1052.2	1039	✓	✓	✓	MS,RI	
68	5-Ethyl-2-furaldehyde[a]	1056.1	1032	×	✓	×	MS,RI	
69	5-Hexanolide	1056.7	1056	✓	✓	×	STD,MS,RI	
70	(*E*)-2-Octenal[a]	1057.6	1056.7	✓	✓	✓	MS,RI	
71	γ-Terpinene	1060	1060	✓	✓	✓	STD,MS,RI	
72	α-Methyl-benzeneacetaldehyde[a]	1065.5	1080	×	✓	×	MS,RI	
73	Acetophenone	1066.8	1067	✓	✓	✓	STD,MS,RI	
74	(*E*)-2-Octen-1-ol[a]	1069.3	1069	✓	✓	✓	MS,RI	
75	2-Methyl-benzaldehyde[a]	1069.3	1067	×	✓	✓	MS,RI	
76	*cis*-Linalool oxide (furanoid)	1071.1	1071	✓	✓	✓	STD,MS,RI	
77	3,5-Octadien-2-one[b]	1071.4	1072	✓	✓	✓	MS,RI	
78	1-octanol	1074.3	1074	✓	✓	✓	STD,MS,RI	
79	2-Acetyl-1-methylpyrrole[a]	1074.9	1096	×	✓	×	MS,RI	
80	Heptanoic acid[a]	1086	1083	×	✓	×	MS,RI	
81	Terpinolene	1087	1088	✓	✓	✓	STD,MS,RI	

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Table 4.2: (*continued*)

No.	Compound name	RI_{Cal}[c]	RI_{Lit}[d]	*GEN*[e]	*MAC*[e]	*SUB*[e]	Identification method[f]	Ref.[g]
82	*trans*-Linalool oxide (furanoid)	1087.4	1087	✓	✓	✓	STD,MS,RI	
83	2,6-Dimethyl-1,7-octadien-3-ol	1087.6	1095	✓	✓	✓	MS,RI	
84	Dimethylaniline[a]	1087.7	1086	✓	✓	✓	MS,RI	
85	2-Nonanone	1089.9	1090	✓	✓	✓	STD,MS,RI	
86	Fenchone[a]	1089.9	1092	×	✓	✓	MS,RI	
87	p-Cymenene	1090	1090	✓	✓	✓	STD,MS,RI	
88	3,5-Octadien-2-one[b]	1093.2	1092	✓	✓	✓	STD,MS,RI	
89	Methyl benzoate[a]	1096.1	1096	✓	✓	✓	MS,RI	
90	Linalool	1101.1	1101	✓	✓	✓	STD,MS,RI	
91	Nonanal	1106.2	1106.5	✓	✓	✓	STD,MS,RI	
92	(*E*)-6-Methyl-3,5-heptadien-2-one	1106.9	1106.3	✓	✓	✓	STD,MS,RI	
93	Hotrienol	1107	1107.7	✓	✓	✓	MS,RI	
94	*trans*-Rose oxide[b]	1111.7	1117	✓	✓	✓	MS,RI	
95	2,6-Dimethyl-cyclohexanol[a]	1112.4	1112	✓	✓	✓	MS,RI	
96	(*E*,*E*)-2,4-Octadienal[a]	1112.4	1113	✓	✓	✓	MS,RI	
97	2,6-Dimethyl-1,3,5,7-octatetraene[a,b]	1114.6	1134	✓	✓	✓	MS,RI	
98	(*Z*)-6-Methyl-3,5-heptadiene-2-one[a]	1115.1	1108	✓	✓	✓	MS,RI	
99	Maltol[a] [3-Hydroxy-2-methylpyrone]	1116.8	1114	✓	✓	✓	MS,RI	
100	α-Cyclocitral[a]	1117.4	1123	✓	✓	✓	MS,RI	
101	2-Phenylethanol	1118.5	1118.4	✓	✓	✓	STD,MS,RI	
102	Thujan-3-one	1120.2	1124	✓	✓	✓	MS,RI	
103	2,6-Dimethyl-1,3,5,7-octatetraene[a,b]	1120.2	1134	✓	✓	✓	MS,RI	
104	Myrcenol[a]	1120.7	1118	✓	×	✓	MS,RI	
105	Isophorone[a]	1120.7	1120	✓	✓	✓	STD,MS,RI	
106	(*E*,*E*)-2,6-Dimethyl-1,3,5,7-octatetraene[a,b]	1123	1134	✓	×	✓	MS,RI	
107	(*E*)-p-Menth-2-en-1-ol[a]	1126.1	1126	✓	×	✓	MS,RI	

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Table 4.2: (*continued*)

No.	Compound name	RI_{Cal}[c]	RI_{Lit}[d]	*GEN*[e]	*MAC*[e]	*SUB*[e]	Identification method[f]	Ref.[g]
108	Methyl octanoate	1125.6	1126	×	×	×	STD	
109	Allo-ocimene	1128.4	1132	×	×	×	STD	
110	2-Ethyl hexanoic acid[a]	1130.4	1128	✓	✓	✓	MS,RI	
111	4-Acetyl-1-methylcyclohexene	1131.6	1131.2	✓	✓	×	STD,MS,RI	
112	2,6-Dimethyl-1,3,5,7-octatetraene[a,b]	1136.8	1137	✓	✓	✓	MS,RI	
113	Unknown 1	1139.7		✓	✓	✓	MS,RI	
114	(*E*)-3-Nonen-2-one	1139.8	1144	✓	✓	✓	STD,MS,RI	
115	Benzyl cyanide[a]	1140.3	1140	✓	×	×	MS,RI	
116	5-Ethyl-6-methyl-3*E*-hepten-2-one[a,b]	1142.4	1143	×	✓	×	MS,RI	
117	Lilac aldehyde A	1142.6	1155	✓	✓	✓	MS,RI	
118	(*E*)-p-Menth-2-en-1-ol[a]	1142.9	1142	✓	×	✓	MS,RI	
119	4-Ketoisophorone	1143.3	1145	✓	✓	✓	STD,MS,RI	
120	3-Phenylbut-1-ene[a]	1148.1	1147.7	✓	✓	✓	MS,RI	
121	Camphor[a]	1148.1	1147	✓	✓	✓	MS,RI	
122	Lilac aldehyde C	1151	1163	✓	✓	✓	MS,RI	
123	Nerol oxide	1153.5	1155	✓	✓	✓	MS,RI	
124	Citronella[a]	1153.5	1153	✓	×	✓	MS,RI	
125	(*E*,*Z*)-2,6-Nonadienal	1153.8	1153	✓	✓	✓	STD,MS,RI	
126	(*Z*)-Ocimenol	1154.1	1155	✓	✓	✓	MS,RI	
127	4-Vinylanisole	1154.3	1153	×	×	×	STD	
128	Menthone[a]	1156.3	1166	×	✓	×	MS,RI	
129	2,3,3-Trimethyl-bicyclo[2.2.1]heptan-2-ol[a]	1156.6	1157	✓	×	✓	MS,RI	
130	(*E*)-2-Nonenal	1159.2	1159.9	✓	✓	✓	STD,MS,RI	
131	Pinocarvone[a]	1162.1	1164.2	✓	×	×	MS,RI	
132	Benzyl acetate	1162.8	1163	✓	✓	✓	STD,MS,RI	
133	iso-Menthone[a]	1164.7	1163	✓	✓	✓	MS,RI	

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Table 4.2: (*continued*)

No.	Compound name	RI_{Cal}[c]	RI_{Lit}[d]	*GEN*[e]	*MAC*[e]	*SUB*[e]	Identification method[f]	Ref.[g]
134	(*E*)-Ocimenol	1165.2	1153	✓	✓	✓	MS,RI	
135	Isoborneol	1165.2	1162	×	×	×	STD	
136	Propiophenone	1165.5	1165	×	×	×	STD	
137	Ethyl benzoate[a]	1170.7	1071	×	✓	×	MS,RI	
138	*cis*-Linalool oxide (pyranoid)	1171.1	1167	✓	✓	✓	MS,RI	
139	1-Nonanol[a]	1173.4	1171	✓	✓	✓	MS,RI	
140	Borneol	1173.6	1175	✓	✓	✓	STD,MS,RI	
141	2-Phenethyl formate[a]	1174.2	1176	✓	✓	✓	MS,RI	
142	3-Methoxy-2-isobutylpyrazine[a]	1175.8	1179	✓	×	✓	MS,RI	
143	3,5-Dimethyl-benzaldehyde[a]	1176.6	1169	✓	✓	✓	MS,RI	
144	*trans*-Linalool oxide (pyranoid)	1176.7	1173	✓	✓	✓	MS,RI	
145	dl-Menthol[a]	1178.9	1178	✓	✓	✓	MS,RI	
146	Terpinen-4-ol	1181.5	1181.5	✓	✓	✓	STD,MS,RI	
147	(3*Z*)-Hexenyl butanoate[a]	1184	1185	×	✓	×	MS,RI	
148	1-Acetyl-4-methylbenzene[a]	1185.1	1183	✓	✓	✓	MS,RI	
149	3,9-Epoxy-p-menth-1-ene[a]	1186.9	1178	✓	✓	✓	MS,RI	
150	p-Cymen-8-ol	1188.4	1183	×	×	×	STD	
151	Octanoic acid[a]	1189.1	1186	✓	✓	✓	MS,RI	
152	2-Decanone[a]	1192.4	1192	✓	✓	✓	MS,RI	
153	Methyl salicylate[a]	1193.4	1193	✓	✓	✓	MS,RI	
154	Ethyl octanoate[a]	1195	1195	✓	×	×	MS,RI	
155	Myrtenal[a]	1195.5	1194	✓	✓	×	MS,RI	
156	α-Terpineol	1195.8	1195	✓	✓	✓	STD,MS,RI	
157	*trans*-p-Menth-8-en-2-one[a]	1198.2	1200	✓	✓	✓	MS,RI	
158	Safranal	1198.3	1197	×	✓	×	MS,RI	
159	(3*E*)-Hexenyl butanoate[a]	1199	1185.6	✓	×	×	MS,RI	

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Table 4.2: (*continued*)

No.	Compound name	RI_{Cal}[c]	RI_{Lit}[d]	*GEN*[e]	*MAC*[e]	*SUB*[e]	Identification method[f]	Ref.[g]
160	Dodecane[a]	1200.4	1200	✓	✓	✓	STD,MS,RI	
161	Benzylidenemalonaldehyde[a]	1202.4	1215	✓	✓	✓	MS,RI	
162	Decanal	1206.8	1206.8	✓	✓	✓	STD,MS,RI	
163	(*E*,*E*)-2,4-Nonadienal[a]	1216.4	1216	✓	✓	✓	MS,RI	
164	4,7-Dimethyl-benzofuran[a]	1216.5	1220.4	✓	✓	✓	MS,RI	
165	p-Menth-1-en-9-al isomer 1	1219.2	1198	✓	×	✓	STD,MS,RI	[5]
166	β-Cyclocitral	1219.2	1219	✓	✓	✓	STD,MS,RI	
167	p-Menth-1-en-9-al isomer 2	1219.2	1200	×	✓	×	STD,MS,RI	[5]
168	7-Methyl-3-methylene-6-octen-1-ol[a]	1219.9	1221	✓	×	×	MS,RI	
169	*trans*-Carveol[a]	1223.2	1223.9	✓	×	×	MS,RI	
170	3-Phenylfuran[a]	1223.3	1226.8	×	✓	×	MS,RI	
171	Methyl nonanoate	1224.9	1224	×	×	×	STD	
172	Nerol	1226	1230	✓	✓	✓	STD,MS,RI	
173	Benzothiazole	1227.2	1227	✓	✓	✓	STD,MS,RI	
174	Citronellol[a]	1228.8	1226	✓	✓	×	MS,RI	
175	(*Z*)-3-Hexenyl isovalerate	1230.9	1235	×	✓	×	STD,MS,RI	
176	(*Z*)-3-Hexenyl-(*E*)-2-butenoate[a]	1234.2	1231	✓	✓	×	MS,RI	
177	3,9-Epoxy-1-p-menthene[a]	1234.2	1236	✓	✓	✓	MS,RI	
178	Linalool hydrate[a]	1236.6	1237	✓	✓	✓	MS,RI	
179	(*E*)-3-Hexenyl isovalerate[a]	1237	1237	×	✓	×	MS,RI	
180	Citral[a]	1240.5	1240	✓	×	×	MS,RI	
181	Neral	1240.5	1238	×	×	×	STD	
182	Carvotanacetone[a]	1246.6	1251	✓	✓	✓	MS,RI	
183	Carvone[a]	1246.7	1243	✓	✓	✓	MS,RI	
184	3-Ethyl-4-methyl-1H-pyrrole-2,5-dione[a]	1248.6	1234.8	✓	×	✓	MS,RI	
185	α-Ionene[a]	1255.2	1255	✓	✓	✓	MS,RI	
186	2-Isopropyl-5-methyl-3-cyclohexen-1-one[a]	1255.8	1251	×	✓	✓	MS,RI	

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Table 4.2: (*continued*)

No.	Compound name	RI_{Cal}[c]	RI_{Lit}[d]	*GEN*[e]	*MAC*[e]	*SUB*[e]	Identification method[f]	Ref.[g]
187	2-Phenethyl acetate	1256.1	1256	✓	✓	✓	MS,RI	
188	Geraniol	1256.4	1253	✓	✓	✓	STD,MS,RI	
189	4-Methoxy-benzaldehyde[a]	1257.8	1258	✓	✓	✓	MS,RI	
190	p-Anisaldehyde	1257.8	1250	×	×	×	STD	
191	β-Homocyclocitral[a] [2,6,6-Trimethyl-1-cyclohexene-1-acetaldehyde]	1258.5	1261.3	✓	✓	✓	MS,RI	
192	Carvenone	1258.8	1258	✓	×	×	MS,RI	
193	Benzyl propanoate[a]	1259	1257	×	✓	×	MS,RI	
194	(*Z*)-2-Decenal[a]	1264.6	1263	✓	✓	✓	MS,RI	
195	2-Hydroxy-3-isopropyl-6-methylcyclohex-2-enone[a]	1267.9	1274.1	×	✓	✓	MS,RI	
196	Geranial	1270.9	1271	✓	✓	✓	STD,MS,RI	
197	2-Phenylbut-2-enal[a]	1271.9	1274.4	✓	✓	✓	MS,RI	
198	4,8-Dimethyl-nona-3,8-dien-2-one[a]	1273.6	1275.5	×	✓	✓	MS,RI	
199	(*E*)-Cinnamaldehyde[a]	1275.9	1270	×	✓	×	MS,RI	
200	Phellandral[a]	1279.9	1276	✓	✓	✓	MS,RI	
201	Nonanoic acid	1284.1	1283	✓	✓	✓	STD,MS,RI	
202	Bornyl acetate[a]	1288.6	1289	✓	✓	✓	MS,RI	
203	Anethole[a]	1289.3	1290	✓	✓	✓	MS,RI	
204	2-Undecanone	1294.7	1295	✓	✓	✓	STD,MS,RI	
205	p-Cymen-7-ol[a]	1297.5	1295	✓	×	×	MS,RI	
206	1-Methyl-naphthalene[a]	1298.5	1299.1	✓	✓	✓	MS,RI	
207	Theaspirane isomer 1[b]	1300.6	1288	×	×	×	STD	[5]
208	Geranyl formate[a]	1300.9	1300	✓	✓	✓	STD,MS,RI	
209	4-(1-Methylpropyl)-phenol[a]	1308.1	1313.7	✓	✓	✓	MS,RI	
210	Carvacrol[a]	1308.4	1306	✓	✓	✓	MS,RI	
211	Undecanal	1309.8	1310	✓	✓	✓	STD,MS,RI	
212	Edulan I[a] [3,5,6,8a-tetrahydro-2,5,5,8a-tetramethyl-, trans-2H-1-Benzopyran]	1312.9	1314	×	✓	×	MS,RI	
213	2-Methoxy-4-vinylphenol[a]	1316.7	1317	✓	×	×	MS,RI	

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Table 4.2: (*continued*)

No.	Compound name	RI_{Cal}[c]	RI_{Lit}[d]	*GEN*[e]	*MAC*[e]	*SUB*[e]	Identification method[f]	Ref.[g]
214	Theaspirane isomer 2[b]	1318.8	1304	×	×	×	STD	[5]
215	(*E*,*E*)-2,4-Decadienal[a]	1319.4	1319	✓	✓	✓	STD,MS,RI	
216	(*Z*)-Hex-3-enyl-(*E*)-2-methylbut-2-enoate	1322	1325	×	×	×	STD	
217	Methyl decanoate	1324.9	1326	×	×	×	STD	
218	Hexyl tiglate	1331	1333	×	×	×	STD	
219	(*E*)-2-Hexenyl tiglate[a]	1337.2	1338	×	✓	×	MS,RI	
220	Piperonal[a]	1340.6	1347	✓	✓	✓	MS,RI	
221	4-Acetylanisole[a]	1354.5	1348	✓	×	×	MS,RI	
222	1,2-Dihydro-1,1,6-trimethyl-naphthalene[a]	1355.5	1355	×	✓	×	MS,RI	
223	Eugenol	1357.8	1358	✓	✓	✓	STD,MS,RI	
224	Nonan-4-olide	1359.6	1358	✓	✓	✓	STD,MS,RI	
225	Megastigma-4,6(*E*),8(*E*)-triene	1361.3	1360	✓	×	×	MS,RI	
226	Butyl carbitol acetate[a] [Ethanol, 2-(2-butoxyethoxy)-, acetate]	1361.7	1361.6	×	✓	×	MS,RI	
227	Neric acid[a]	1363.8	1365	✓	×	×	MS,RI	
228	2-Undecenal[a]	1364.5	1368	✓	✓	✓	MS,RI	
229	4-*tert*-Butylcyclohexyl acetate[a]	1367.4	1368	×	✓	×	MS,RI	
230	Phenylethyl alcohol[a]	1371.9	1374	✓	×	×	MS,RI	
231	Butyl benzoate[a]	1373.9	1377	×	×	×	STD	
232	n-Decanoic acid[a]	1374.2	1374	✓	✓	✓	MS,RI	
233	Benzoic acid, 4-methoxy-, methyl ester[a]	1375	1373	✓	×	×	MS,RI	
234	α-Copaene	1376.3	1376	×	×	×	STD,MS,RI	
235	Geranyl acetate	1376.6	1376	✓	✓	×	STD,MS,RI	
236	(*E*)-β-Damascenone	1379.8	1380	✓	✓	✓	STD,MS,RI	
237	Methyl cinnamate[a]	1383.9	1389	✓	×	×	MS,RI	
238	β-Bourbonene	1385.4	1374	✓	×	×	MS,RI	
239	10-(acetylmethyl)-3-Carene[a,b]	1386.4	1387	✓	×	×	MS,RI	[23]
240	2-Dodecanone[a]	1391.6	1391	✓	×	×	MS,RI	

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Table 4.2: (*continued*)

No.	Compound name	RI_{Cal}[c]	RI_{Lit}[d]	*GEN*[e]	*MAC*[e]	*SUB*[e]	Identification method[f]	Ref.[g]
241	Methyleugenol[a]	1398.6	1399	✓	×	×	MS,RI	
242	Tetradecane[a]	1400.5	1400	×	×	×	STD	
243	6,10-Dimethyl-2-undecanone[a]	1400.7	1400	×	✓	×	STD,MS,RI	
244	3-Hydroxy-p-anisaldehyde[a]	1404.1	1401	✓	×	×	STD,MS,RI	
245	2,6-Dimethyl-naphthalene[a]	1404.9	1408	×	×	×	STD	
246	Dodecanal	1407.4	1409	✓	✓	✓	STD,MS,RI	
247	(*E*)-β-Damascone	1407.7	1408	✓	✓	✓	STD,MS,RI	
248	2,4,7,9-Tetramethyl-5-decyn-4,7-diol[a]	1408.9	1407.3	✓	✓	✓	MS,RI	
249	(*E*)-Caryophyllene	1420.7	1419	×	×	×	STD	
250	α-Ionone	1421.1	1426	✓	✓	✓	STD,MS,RI	
251	1,7-Dimethyl-naphthalene[a]	1421.6	1419	✓	×	×	MS,RI	
252	Geranyl acetone	1447.6	1448	✓	✓	✓	MS,RI	
253	Isoeugenol[a]	1450.7	1452	✓	×	×	MS,RI	
254	α- Humulene	1457.4	1455	×	×	×	STD	
255	*cis*-β-Santalene[a]	1460.7	1461	✓	×	×	MS,RI	
256	Undecanoic acid[a]	1464.4	1465	✓	×	×	MS,RI	
257	(*E*)-β-Ionone	1477.8	1477	✓	✓	✓	STD,MS,RI	
258	3,4-Dehydro-γ-ionone	1478	1485	×	✓	×	MS,RI	
259	5,6-Epoxy-β-ionone	1481.3	1497	×	×	×	STD	
260	Phenethyl isovalerate[a]	1484.5	1489	✓	×	×	MS,RI	
261	5-Methyl-2-phenyl-2-hexenal[a]	1484.9	1486	✓	✓	×	MS,RI	
262	Apocynin[a] [1-(4-Hydroxy-3-methoxyphenyl)ethanone]	1488.5	1480	✓	×	×	MS,RI	
263	5-Decanolide[a]	1492.1	1492	✓	×	✓	STD,MS,RI	
264	Pentadecane	1500.5	1500	×	×	×	STD	
265	α-Muurolene	1500.7	1499	✓	×	×	MS,RI	
266	*trans*-calamenene[a]	1500.7	1508	✓	✓	✓	MS,RI	
267	Butylated Hydroxytoluene[a]	1501	1504	✓	✓	×	MS,RI	

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Table 4.2: (*continued*)

No.	Compound name	RI_{Cal}[c]	RI_{Lit}[d]	*GEN*[e]	*MAC*[e]	*SUB*[e]	Identification method[f]	Ref.[g]
268	2,4-bis(1,1-dimethylethyl)-Phenol[a]	1506.2	1512	✓	✓	×	MS,RI	
269	Dihydroagarofuran	1507.4	1504	✓	×	×	MS,RI	
270	Dibenzofuran[a]	1515.6	1517	✓	✓	×	MS,RI	
271	Methyl dodecanoate	1520.7	1526	✓	×	×	STD	
272	Calamenene	1520.8	1521	✓	×	×	MS,RI	
273	6,10-dimethyl-(*E*,*Z*)-3,5,9-Undecatrien-2-one[a]	1524.6	1535	✓	✓	×	MS,RI	
274	Lilial[a]	1524.6	1535	✓	✓	×	MS,RI	
275	Dihydroactinidiolide	1529.4	1525	✓	×	✓	MS,RI	
276	Calamenene[a]	1540.8	1536	✓	×	×	MS,RI	
277	α-Calacorene	1540.9	1541	✓	✓	×	MS,RI	
278	germacrene B[a]	1541.1	1556	✓	×	×	MS,RI	
279	3,3,5,6-Tetramethyl-1-indanone[a]	1541.7	1555	×	✓	×	MS,RI	
280	Mellein[a]	1543.7	1549	×	✓	×	MS,RI	
281	(*E*)-Nerolidol	1557.7	1563	×	×	×	STD	
282	Dodecanoic acid	1560.5	1559	✓	✓	×	STD,MS,RI	
283	(*Z*)-3-Hexenyl benzoate	1568	1580	×	×	×	STD	
284	Megastigmatrienone	1571.6	1591	✓	✓	✓	MS,RI	[24]
285	Spathulenol[a]	1577.9	1578	×	✓	×	MS,RI	
286	(*E*,*E*)-6,10-dimethyl-3,5,9-Undecatrien-2-one[a]	1578	1589	✓	✓	×	MS,RI	
287	Caryophyllene oxide	1581	1581	✓	✓	×	MS,RI	
288	Globulol[a]	1584.5	1584	×	✓	×	MS,RI	
289	Epicedrol[a]	1605.2	1608	✓	×	×	MS,RI	
290	Cedrol[a]	1605.2	1601	✓	✓	✓	MS,RI	
291	3-Hydroxy-β-damascone[a]	1607.8	1617	✓	✓	×	MS,RI	
292	Isopropyl dodecanoate[a]	1620	1618	✓	✓	×	MS,RI	
293	γ-Eudesmol	1620.5	1620	✓	×	✓	MS,RI	

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Table 4.2: (*continued*)

No.	Compound name	RI_{Cal}[c]	RI_{Lit}[d]	*GEN*[e]	*MAC*[e]	*SUB*[e]	Identification method[f]	Ref.[g]
294	Benzophenone[a]	1626.2	1625	✓	✓	✓	MS,RI	
295	3-Oxo-α-ionol[a]	1639.2	1630	✓	✓	×	MS,RI	
296	α-Cadinol	1639.9	1640	✓	✓	×	MS,RI	
297	Gingerone[a]	1641.8	1645	✓	✓	✓	MS,RI	
298	Methyl dihydrojasmonate[a]	1644.1	1648	✓	×	✓	MS,RI	
299	β-Eudesmol	1655.4	1651	×	×	×	STD	
300	3-Keto-β-ionone[a]	1656.5	1661	✓	×	✓	MS,RI	
301	Cadalene[a]	1670.7	1671	✓	×	✓	MS,RI	
302	α-Bisabolol	1686	1683	×	✓	×	MS,RI	
303	2,6-Diisopropylnaphthalene[a]	1716.7	1716.8	×	✓	×	MS,RI	
304	Tetradecanoic acid[a]	1757.3	1758.2	✓	✓	✓	MS,RI	
305	Benzyl benzoate[a]	1764.4	1765.9	✓	✓	✓	MS,RI	
306	Isopropyl myristate	1822.1	1812	×	×	×	STD	
307	Hexahydrofarnesylactone	1839.2	1845	×	×	×	STD	

[a] Compounds identified in honeybush tea for the first time
[b] The stereochemistry of the compound was not determined
[c] Calculated retention index
[d] Retention index (RI) from literature
[e] GEN: *C. genistoides*, MAC: *C. maculata* and SUB: *C. subternata*
[f] Methods used for identification of compounds; STD: reference standards, MS: mass spectral and RI-retention index data
[g] References used to extract retention index (RI) data

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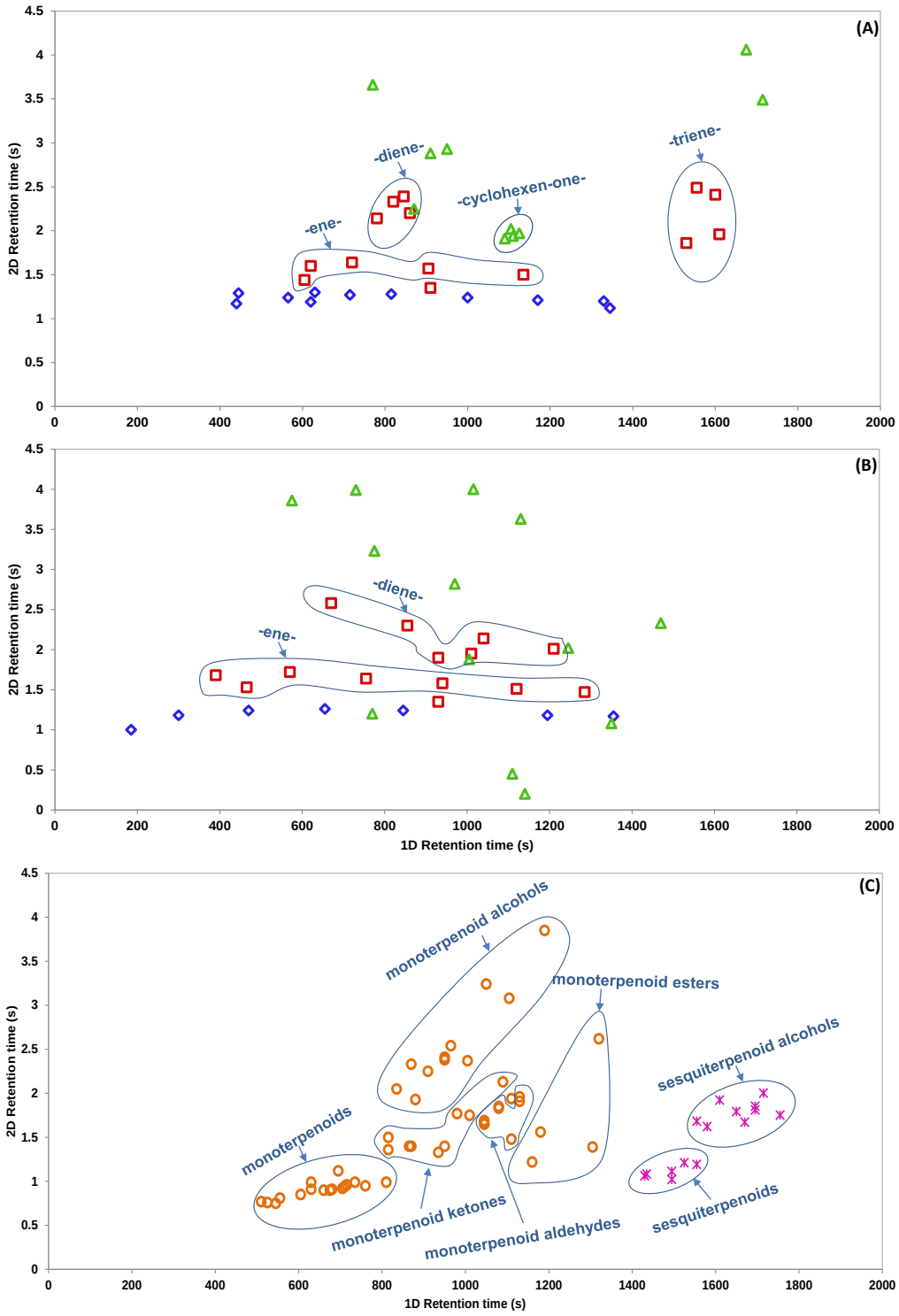

Figure 4.6: Contour plots illustrating the grouping of volatile compounds of honeybush tea in the 2-dimensional separation space. (**A**) ketones, (**B**) aldehydes and (**C**) terpenenoids. Symbols represent the following classes: blue diamonds (◊): saturated volatile compounds, red squares (□): unsaturated volatile compounds, green triangles (Δ): aromatic volatile compounds, orange circles (o): monoterpenoids and pink stars (⁎) sesquiterpenoids.

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One of the advantages offered by GC×GC is that structured contour plots are often obtained, where one will find compounds belonging to the same class grouped together [21,22]. Owing to the complexity of the sample, no clear indication of such grouping is evident from the GC×GC contour plots for the honeybush tea samples *(cf.* **Figure 4.5**). By extracting the retention time (t_R) data for different classes of compounds, 2D chromatograms can be plotted separately to clearly present the elution patterns. **Figure 4.6** shows an example of the partially structured two-dimensional (2D) plots obtained for terpenoids, ketones and aldehydes found in honeybush tea.

Saturated ketones and aldehydes were characterised by low second dimension retention times (^2t_R), but eluted within distinct first dimension retention time (^1t_R) windows, whereas unsaturated ketones and aldehydes have a higher ^2t_R (*cf*. **Figure 4.6A** and **B**). For unsaturated compounds clusters according to the number of double bonds were observed: compounds containing two double bonds (-diene-) showed higher ^2t_R than single double-bonded compounds (-ene-). Compounds containing three double bonds (-triene-) eluted later in the ^{1}D (high ^1t_R), but had similar ^2t_R-times as the –dienes- (**Figure 4.6A** and **B**)**.** Aromatic aldehydes were spread widely across the 2D chromatographic space and showed no clear clustering. In contrast, the ketones containing a cyclohexenone backbone were found grouped in the centre of the 2D chromatogram, whereas the aromatic ketones had higher ^2t_R-times. Terpenoids were grouped into monoterpenoids and sesquiterpenoids based on their two-dimensional retention. Five groups of monoterpenoids can be distinguished based on the relative retention in both dimensions: monoterpene hydrocarbons (monoterpenes) were less retained in the ^{2}D than the oxygen containing monoterpenoids. Monoterpenoids eluted in the ^{1}D in the following order: ketones eluted first, followed by aldehydes and esters. Similarly, due to the presence of the –OH group in sesquiterpenoids alcohols, they were retained slightly longer on the Stabilwax column than the sesquiterpenoids. This brief discussion shows that, despite the highly complex nature of honeybush tea volatiles, compounds were somewhat orderly distributed according to their nature owing to the orthogonal separation mechanisms used in GC×GC.

4.4. Conclusions

The data reported herein not only confirms the separation power of GC×GC, in this case using a commercial cryogenic modulator, but also demonstrates the amount of information this technique can provide when coupled to a powerful detector such as TOF-MS. GC×GC-TOF-MS enabled the identification of 158 new volatile compounds in honeybush tea, which could not be attained by 1D GC and GC×GC-qMS. In total 272 compounds were identified in the three *Cyclopia* species *C. genistoides*, *C. maculata* and *C. subternata*, and significant differences in the volatile composition between species was observed. 232, 217 and 181 compounds were identified in *C. genistoides C. maculata*, and *C. subternata*, respectively. Some compounds seem to be species-specific, although this should be confirmed by analysing a larger batch of samples. Especially the observation that (*E*)-cinnamaldehyde was detected only in *C. maculata* samples is noteworthy, since this may in part be responsible for the unique sensory profile of honeybush tea produced from this species. In future, quantitative analysis should be done to confirm the sensory contribution of the compounds identified. Multivariate data analysis of the GC×GC-TOF-MS data would also be interesting to investigate in more detail the differences

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between the *Cyclopia* species. The method reported here could be used to identify marker compounds of honeybush species and could in future be used as a quality control tool.

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4.5. References

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Chapter 5

General conclusions and Recommendations

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5.1. General conclusions and recommendations

The analysis of the complex volatile fractions of natural products presents a severe analytical challenge. This is also true for honeybush tea, which contains of a wide range of volatile compounds differing in their physiochemical properties and concentrations. One-dimensional gas chromatography (GC) is the method of choice for the analysis of volatiles, and has been applied with some success to honeybush analysis. However, for such a complex sample, the performance of capillary GC is insufficient for complete separation of all compounds. The main aim of this study was therefore to develop comprehensive two-dimensional gas chromatographic (GC×GC) methods for the improved separation of honeybush tea volatile compounds.

The main part of this study entailed the use of a new single-stage thermal modulator, its evaluation and the optimisation of a GC×GC method for honeybush analysis (**Chapter 3**). Headspace-solid phase micro-extraction (HS-SPME) was used in combination with GC×GC separation and flame ionisation detection (FID) and quadrupole mass spectrometry (qMS) for the analysis of two sets of honeybush samples (different harvest seasons) of which each consisted of 15 samples. Method development involved systematic optimisation of chromatographic conditions, including various column combinations, modulation settings and oven temperature and carrier gas flow rates. A non-polar × polar column combination was found to provide the best 2D separation of honeybush tea volatiles. The performance of the thermal modulator was found to be significantly affected by the desorption temperature (discharge voltage). Therefore, a compromise had to be made between t obtaining good separation and minimising analyte breakthrough. Nevertheless, the optimised method provided good separation of honeybush tea volatiles, and a total of 69 compounds were identified using references standards. Hyphenation to qMS did not allow identification of more than two additional compounds due to the slow acquisition rate of this detector. Owing to the highly reproducible performance of this modulator, multivariate data analysis in the form of principle component analysis (PCA) was facilitated. This allowed investigation of the difference between the three *Cyclopia* species studied (*C. genistoides*, *C. subternata* and *C. maculata*). Significant difference in the volatile profiles between the three species was observed for first sample set. Interestingly, *C*. *maculata* showed a distinct volatile composition compared to the other two species - this was in accordance with sensory data for the same set of samples. The compounds responsible for the unique sensory properties of *C. maculata* could however not be identified due to the limitations of qMS detection. Furthermore, no distinction was made between the three species for the second set of sample. Overall, it can be concluded that the new single-stage thermal modulator provides a useful, affordable alternative to cryogenic GC×GC instruments.

In the second part of the study (**Chapter 4**), a commercial GC×GC instrument equipped with a cryogenic modulator and hyphenated to time-of-flight mass spectrometry (TOF-MS) was used to obtain more detailed qualitative information regarding honeybush tea volatiles. This configuration provided a wealth of information. Due to the improved separation and sensitivity offered by GC×GC coupled to the high speed acquisition and deconvolution capabilities of the TOF-MS, a total of 272 compounds were identified in the same set of honeybush samples. Of these, 73 were identified using reference standards, and the remainder were tentatively

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identified using MS spectra and retention indices (RIs). In *C. genistoides*, C*. maculata* and *C. subternata* 232, 217, and 181 compounds were identified, respectively. 158 compounds were tentatively identified in honeybush tea for the first time. Significantly, (*E*)-cinnamaldehyde was uniquely detected in *C. maculata* samples, which points to this compound as being likely responsible for the characteristic cassia/cinnamon aroma of this species identified by sensory analysis.

In light of the results attained in this work, it can be concluded that GC×GC is a promising technology for the more detailed chemical analysis of honeybush tea volatiles. The single-stage thermal modulator used in this study offered consumable-free operation and showed good performance. The limitations of qMS used in this work limited the number of compounds that could be identified. Future work on the hyphenation of this configuration to TOF-MS would therefore be interesting; ultimately this could serve as an alternative and more affordable GC×GC system. The excellent reproducibility observed for this system, and the compatibility of GC×GC-FID data with multivariate data analysis techniques, implies that this approach shows promise as a quick scanning tool for quality control of honeybush tea. Future studies should also include quantitative analysis of honeybush tea, likely using FID detection, and relation of these data to odour activity values in the tea. The GC×GC-TOF-MS results confirm the power of this technique for natural product analysis. Future work in this regard should involve analysis of additional *Cyclopia* species being considered for commercial cultivation, as well as the analysis of a larger set of *C. maculata* samples to confirm the initial findings reported here that this species is unique in its containing (*E*)-cinnamaldehyde.