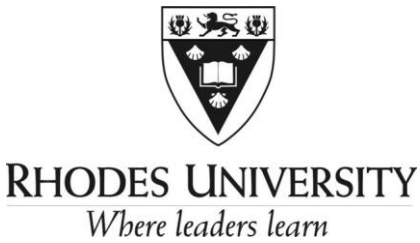

Profiling For Volatile Compounds in the Kgalagadi Desert Truffle

A thesis submitted in fulfilment of the requirement for

The degree of

Master of Science in Chemistry

By

Juda Bogopa

Supervised by

Professor Nelson Torto

Dedication

To my wife and kids

Acknowledgements

Prof. Nelson Torto: Thank you for bringing me aboard. Words will never be enough to express my gratitude to you. Your networking and collaborative skills, both locally and internationally are so inspiring, only for one to wish they could belong to the pool that will keep that wheel rolling for the betterment of research and quality of life in Africa. The many forms of support you have granted will go a long way in making me understand the true meaning of leadership. Studying at a university which goes by a motto “where leaders learn”, under your leadership has been so intriguing. Thanks boss!

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Prof. Charlotta Turner: My gratitude to you. Thank you for your kind invitation for me to pursue my research work at Lund University, Sweden. Working under your supervision and interaction with your research group has been such an experience. Through you I would like to thank STINT collaboration for the sponsorship. My regards to the whole Green Technology Group.

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Finally, to my kids; Tlhalefo, Lebogang and Ryan! Thanks for staying intact while dad was away.

“I Thank God the almighty for having blessed this journey.”

Abstract

The thesis focuses on 'green' sample preparation techniques that utilise minimal or no organic solvents thereby producing negligible volumes of organic waste, to ensure safety both to user and the environment. Volatile compounds were extracted and pre-concentrated from Kgalagadi desert truffles (*kalaharituber*) by headspace solid phase microextraction (HS-SPME) and supercritical fluid extraction (SFE). PHWE was employed for the extraction of amino acids and fatty acids. Subsequent analysis of volatile compounds was carried out by gas chromatography coupled with mass spectrometry. Four types of HS-SPME fibers (PDMS 100 µm, PDMS 7 µm, Polyacrylate 85 µm, CAR/DVB/PDMS 50/30 µm) were evaluated. A total of 24 volatile compounds with a molecular weight range from 110 to 354, the most prominent peak being 2-t-Butyl-2,3-dimethyl-3-buten-1-ol ($C_{10}H_{20}O$, MW 156) were detected after sampling with a PDMS 100 µm fiber. Less volatile compounds were detected after SFE with CO_2. A total of 16 amino acids were identified while 17 fatty acids (MW from 132.12 to 367.49) were also identified. The characteristic profile of the Kgalagadi desert truffle was found to contain mainly fatty acid methyl esters and unsaturated aliphatic hydrocarbons. The most prominent compound peaks identified were; 2-t-butyl-2,3-dimethyl-3-buten-1-ol, disulfide, ethyl benzoic acid 2-4-dhydroxy-3,6-dimethyl-methyl ester, 8,11-octadecanoic acid methyl ester, benzoic acid, 2,4-dihydroxy-3,6-dimethyl-methyl ester, isoquinoline, 1 butyl-3,4-dihydro and 3-heptanone, 6 methyl. Optimization results indicated that fresh slices from the heart of truffles were the best to use for HS-SPME-GCMS volatile compound analysis as they showed a higher sensitivity.

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List of Abbreviations

AFM	Atomic force microscope
BCA	Botswana college of agriculture
CAR/DVB/PDMS	Carboxen/Divinylbenzene/Polydimethylsiloxane
CW	Carbowax
CW-DVB	Carbowax-Divinylbenzene
DHE	Dynamic headspace extraction
D-HS	Dynamic headspace
DVB	Divinylbenzene
EC	Electrical conductivity
EcM	Ectomycorrhiza
EI	Electron ionisation
ESI	Electron spray ionisation
EPA	Environmental protection agency
FID	Flame ionisation detector
FTIR	Fourier transform infra - red
HCC-HS	High concentration capacity – headspace
HPLC	High performance liquid chromatograph
HS	Headspace
HSAS	Headspace auto sampler
HSGC	Headspace gas chromatograph
HS—LPME	Headspace liquid phase microextraction
HSSE	Headspace sorptive extraction
HS-SPME	Headspace-solid phase microextraction
HS-SPME-GCMS	Headspace-solid phase microextraction-gas chromatography mass spectrometry
INCAT, HS-SPDE	Inside needle capillary adsorption trap headspace

	solid phase dynamic extraction
IS-SPDE	In solution solid phase dynamic extraction
LCA	Life cycle assessment
MESI	Membrane extraction sorbent interface
MHE	Multiple headspace extraction
MME	Membrane microextraction
NFTRC	National food technology research centre
PA	Polyacrylate
PDMS	Polydimethylsiloxane
PHWE	Pressurized hot water extraction
Ppb	Parts per billion
Ppt	Parts per trillion
PTV	Programmed temperature vaporisation
RP-HPLC	Reverse phase high performance liquid chromatograph
SBSE	Stir bar sorptive extraction
SEM	Scanning electron microscope
SFE	Supercritical fluid extraction
SHE	Static headspace extraction
S-HS	Static headspace
SPACE	Solid phase aroma concentrates extraction
SPE	Solid phase extraction
SPE-GC-EIMS	Solid phase extraction-gas chromatography-electron ionisation mass spectrometry
SPME	Solid phase microextraction
SVE	Solvent vapour exit
TPR	Templated resin
UV	Ultraviolet
VOCs	Volatile organic compounds

Chapter 1

1 Introduction

1.1 Background

Diaz et al. [1] suggested that it is important to develop methods that allow for an objective evaluation of truffle aroma. These could help in truffle species identification, product authentication and studying the effect of different growing parameters [1]. Truffle trade has been marred by unfair practices of adulteration; cheap truffles are often made to mimic the expensive black truffle aroma for the European market [1]. A documented profile of the Kgalagadi desert truffle aroma compounds will not only guard against adulteration but also give the Kgalagadi desert inhabitants the market worth of the product and possibly help improve their socio-economic status [2]. Taylor et al. [3] has noted that desert truffles are among the most sought after due to their unique aroma. However, there is very limited research information available on Kgalagadi desert truffle. Little is known about their biology and ecology [3].

The National Food Technology Research Centre (NFTRC) Botswana, has since embarked on a project with the objective of improving preservation techniques and monitoring the effect of processing on the aroma parameters of the Kgalagadi desert truffle [2].

The findings of the project are expected to feed into the NFTRC project [2] (*Propagation and Processing of the Desert Truffle in Botswana*) involving institutional collaboration between the Botswana College of Agriculture (BCA) and NFTRC.

The focus of the thesis was thus on analysis of volatile compounds of the Kgalagadi desert truffle employing 'green chemistry' sample preparation techniques that utilise minimal or no organic solvents thereby producing negligible volumes of organic waste. The techniques used within the scope of the study included; headspace solid phase micro extraction (SPME), supercritical fluid extraction (SFE), pressurised hot water extraction (PHWE), fourier transform infrared spectroscopy (FTIR), scanning electron microscopy, liquid chromatography mass spectrometry (LC-MS), gas chromatography (GC) and gas

chromatography mass spectrometry (GCMS) for extraction, separation, detection and identification of compounds respectively.

1.1.1 Description of truffles

The word truffle in latin means tuber [4]. In French a truffle is known as Truffe, in Spanish Trufa, in German Trüffel, and in Dutch Truffel. Truffles form mychorrhizal relationships with roots of other plants. Mycorrhizae are divided into two groups; ectomycorrhizal and endomycorrhizas. Ectomycorrhiza (EcM) are formed between the roots of woody plants and fungi. All truffles are ectomycorrhizal hence they are always found in close association with trees [3]. Truffles take between 6 to 9 months to mature fully as shown in the truffle life cycle (Fig 1.1.1.1).

The Kgalagadi truffle, has long been classified within the Mediterranean genus *Terfezia* until DNA sequencing showed it to belong to a different evolutionary trajectory, hence it was assigned to the new genus *kalaharituber* [5].

Research has [6] suggested that the organoleptic differences seen over the geographical area of truffles can probably be explained by environmental variation rather than by genetic factors. Trappe et al. [5] discussed that the genera and species of desert truffles differs from continent to continent, many aspects of their ecology are similar, as are their uses by geographically distant, nomadic but culturally unrelated desert peoples [3].

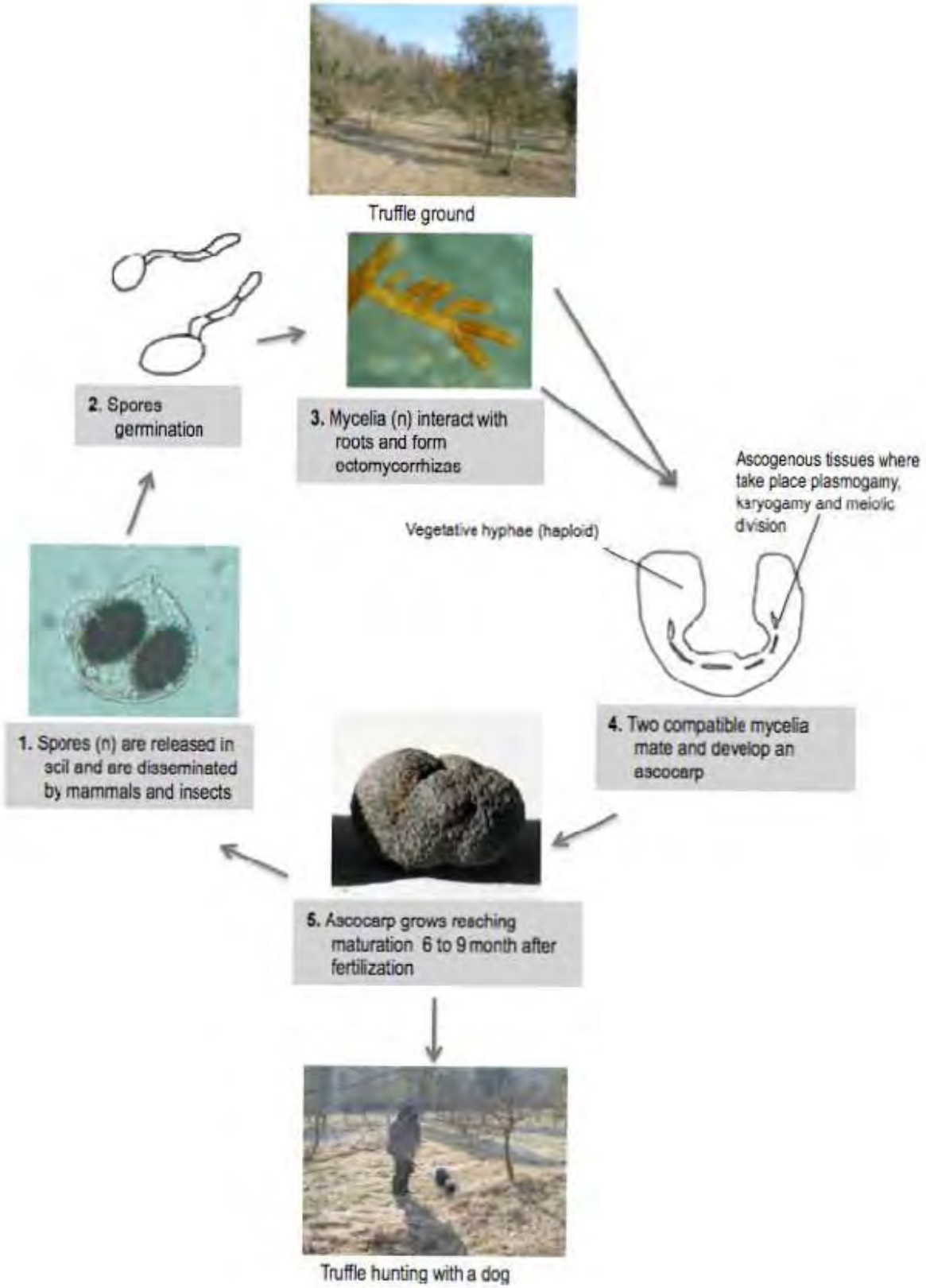


Fig. 1.1.1.1 Truffle life cycle [8]

Fig. 1.1.1.2 A picture showing Kgalagadi desert truffles [4]

The fruiting body of the truffle (Fig 1.1.1.2) is referred to as the ascoma, this is the part that is taken as food, and it is highly priced [4]. Fruiting bodies are large, spherical, thick walled and solid. Those which grow on the ground e.g. mushroom, are referred to as epigeous and those that grow beneath the ground e.g. desert truffle, are said to be hypogeous [4]. Truffles generally grow from a few centimetres across and weigh 30 – 300 g [4]. The size of the Kgalagadi truffle is said to range from 10 – 200 g [3].

Truffles exist in mychorrhizal associations with the roots of other plants [3]. They consist of a hyphal sheath or mantle covering the root tip and a hartig net of hyphae surrounding the plant cells within the root cortex. The hyphae may penetrate the plant cells and in that case the truffle is referred to as ectoendomycorrhizia. The fungal mycelium normally forms an extensive network within the soil and the leaf litter just outside the roots [3]. Ectomycorrhiza are found around 10% of plant families including members of birch, dipterocarp, eucalyptus, oak, pine, and rose families [4]. Truffles rely upon consumption by animals for spore dispersion as opposed to the air currents that disperse spores for epigeous bodies [5].

In a mychorrhizal association, the fungus may colonize the roots of a host plant either intracellularly or extracellularly [4]. This mutualistic relationship forms a very important part of soil life. The host plant constantly provides the fungus with products of photosynthesis; monosaccharides such as glucose and sucrose. On the other hand the plant uses the large surface area of fungi to absorb water and mineral nutrients from the soil. This improves the

mineral absorption capacity of the roots. In soils of basic pH, where phosphate ions are relatively immobilised, the mycelium of the mychorrhizal fungus can access these phosphorus sources and make it available to the plant roots. The ectomycorrhizal fungus (laccari bicolor) is known to lure and kill springtails to obtain nitrogen [6], which is in turn transferred to the host plant. Due to their small diameter, mychorrhizal mycelia can explore a greater volume of soil, providing a larger surface area for absorption. The cell membrane chemistry of the fungi is different from that of plant cells, this makes the mychorrhizae very beneficial to the plants especially in nutrient poor soils. The other benefit to the host plant is that mychorrhizal plants are often resistant to diseases, such as those caused by microbial soil-borne pathogens [7]. They also tend to be more drought resistant.

1.1.2 Availability and natural distribution

Desert truffles are found in arid to semi-arid areas of the world including; Kgalagadi desert (Fig 1.1.2.1), Mediterranean basin, Iraq, Kuwait, Sahara desert, Saudi Arabia, Hungary, Yugoslavia and China [4]. They are very rare. They are seasonal with the harvest time in Botswana being after rains around April. There is no clear information as to the best period of sampling because the truffle yield in Botswana has generally gone down due to factors like over grazing and erratic rainfall [7].

Soil samples from the truffle sites in Botswana have a pH below neutral and low calcium concentrations as opposed to reports from other parts of the world (France and west Africa) where concentrations of calcium around the truffle sites were found to be around 70 % and pH more than neutral [7]. The recommended soil fertility analysis parameters are thus; moisture, carbon dioxide, pH, oxygen, temperature, organic content, nitrogen, available phosphorus, electrical conductivity (EC), exchangeable cations (CEC); potassium, calcium, magnesium and sodium.

Fig. 1.1.2.1 Map of Kgalagadi desert -900 000 km^2 [9]

In Europe, the black truffle has been cultivated due to the high economical and gastronomical value [7]. The Ben Hur research station has also been doing propagation studies and microscope identification of the desert truffles in Namibia (Omaheke region) [2]. There are some farms in the Gantsi area of Botswana for cultivation of the Kalahari desert truffle and studies are in progress at the Botswana College of Agriculture for optimisation of the propagation parameters [2].

1.1.3 Uses of desert truffles

March et al. [10] indicated that truffles find their use in culinary applications in countries such as Spain, France and Italy where they are consumed the most. Truffles are usually served raw by grating onto other dishes like pasta, pizza, omelette, and salads [10].

Traditionally the Bushmen of the Kgalagadi would include truffles in their cooking and regard them very special as it took only trained Bushmen to find truffles [2]. Truffles are believed to have other properties such as treating eye infections, sugar diabetics and have been used for bird catching [4]. In the Kgalagadi region of Botswana, the inhabitants used to preserve truffles by roasting in hot ashes followed by sun drying for a few days [2].

The National Food Technology Research Centre in Botswana has since embarked on a project to diversify and preserve the product range of truffles. Several products of modern use like "truffle butter, canned truffles in olive oil and truffles in brine" have since been developed [2].

The commercial importance of truffles cannot be overlooked as Italian truffles can cost US$2200 per kilogram [4]. The price of the desert truffle per kilogram still needs to be well cited as this rare delicacy seems to be under priced at US$ 26.75 per kilogram [7]. There are currently no commercial producers of the product in Botswana [2].

The commercial importance of desert truffles has necessitated research to find out how truffles differ in quality from those around the world especially the critical parameters of aroma profile, hence the scope of this study.

1.2 Scope of the thesis

The study addressed the analysis of volatile organic compounds composition of the Kgalagadi desert truffle aroma [8]. The aim of the thesis was to evaluate and present the optimal sampling and sample handling techniques associated with truffle sample analysis. Therefore the thesis will discuss the extraction techniques and analytical systems employed to profile the volatile compounds. Thus the scope of the project was to address:

(i) The physical characterization of the Kgalagadi desert truffle morphology by scanning electron microscopy.

(ii) The analysis of extracts by FTIR to profile functional group components of truffle oil and PHWE extracts.

(iii) The effectiveness of green technology extraction yield of truffle volatiles for a method based on supercritical fluid extraction.

(iv) The efficiency of extraction of volatiles employing headspace solid phase microextraction.

(v) The profile of bioactive compounds of the truffle oil and identify the composition of PHWE extracts by LCMS or GCMS.

Chapter 2

2.0 Analysis of volatile compounds

From an analytical point of view, volatile organic compounds (VOCs) can be defined as organic compounds whose vapour pressures are greater than or equal to 0.1 mmHg at 20 °C [11]. The U.S. Environmental Protection Agency (EPA) defines VOCs as "any compound of carbon, excluding carbon monoxide, carbon dioxide, carbonic acid, metallic carbides or carbonates, and ammonium carbonate, that participates in atmospheric photochemical-reactions. Since most VOCs are environmental pollutants, the EPA definition is meant for regulatory purposes [11]. This chapter introduces the topic of volatile compounds; their origin, methods of extraction and analysis.

2.1 Origin of VOCs in truffles

Truffle aroma is made of hundreds of volatiles that vary in concentration and composition depending on the species maturity and origin [1, 5, 8, 10]. The volatile components of truffles have been traced back to a series of biochemical reactions within the fruiting body of the truffle [12]. Discrimination of the truffle fruiting body versus mycelia aromas have been studied using stir bar sorptive extraction [12]. This confirmed published genomic work [8] suggesting that most truffle volatile compounds originated within the fruiting body, the reproductive stage of truffles. Several isoprenoids (terpenoids) have been identified among VOCs produced by ripe *T.borchii* fruiting bodies. Isoprenoids belong to a vast group of secondary metabolites synthesized from isopentenyl diphosphate (IPP), which includes flavor enhancers and fragrances [8]. The Ehrlich pathway (Fig 2.1.1) presents major constituents of truffle aroma containing alcohols that are produced by yeast during amino acid catabolism.

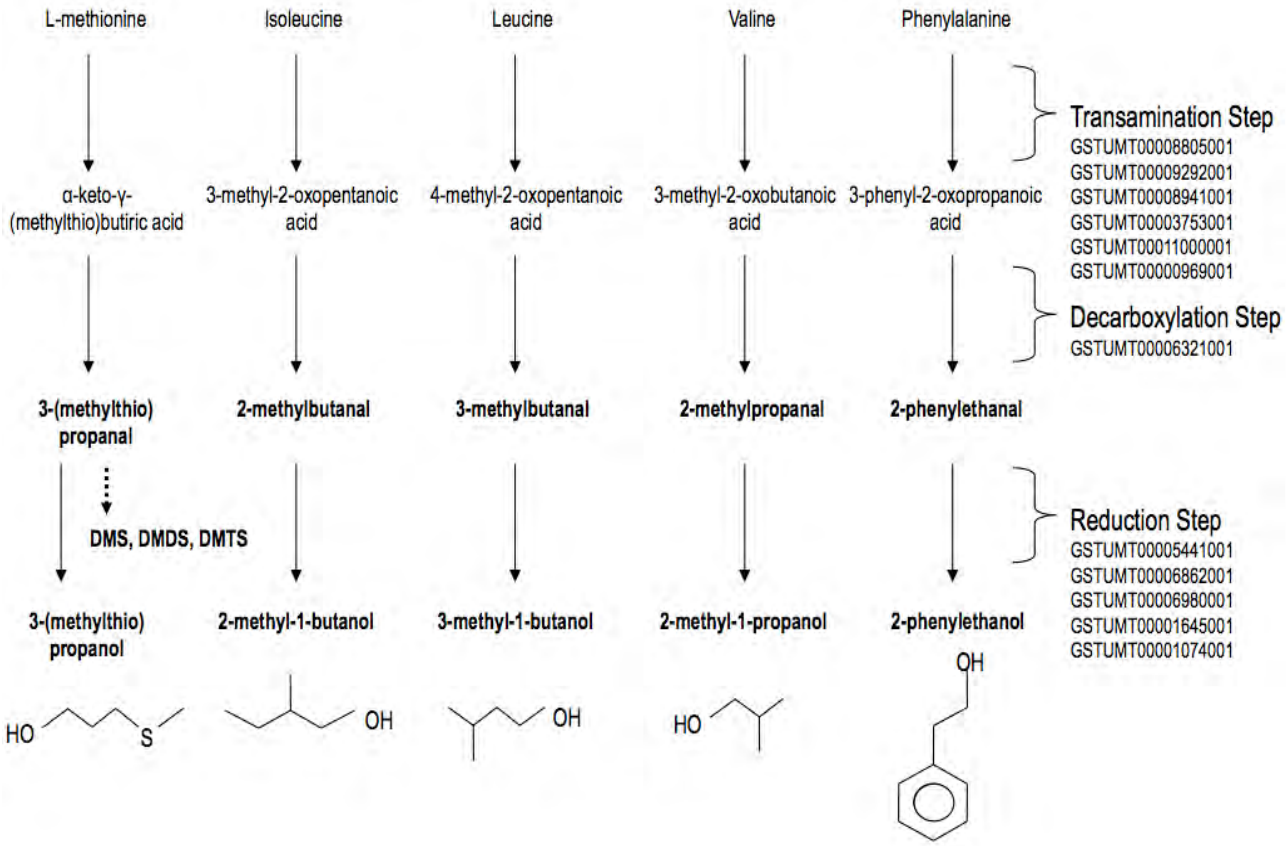


Fig. 2.1.1 Predicted Ehrlich pathways leading to characteristic truffle volatile organic compounds (VOCs) [8].

Based on known yeast pathways, the catabolism of five amino acids could lead to the formation of the aldehydes and alcohols that are key contributors of the truffle aroma. Compounds with a high volatility are in bold. Genes identified in *T. melanosporum* potentially involved in the Ehrlich pathway (transamination, decarboxylation and reduction steps) are listed on the right part of Fig 2.1.1. The formation of dimethyl sulfide (DMS), dimethyl disulfide (DMDS) and dimethyltrisulfide (DMTS) from 3-(methylthio) propanal (dashed arrow) could occur through chemical non-enzymatic degradation [8].

Earlier studies suggested that truffle aroma as a whole could be a mixture of compounds produced by both mycelium and fruiting body associated microorganisms. However a set of genes homologous to the Ehrlich pathway (Fig 2.1.1) showed many of them expressed in the

fruiting body suggesting that truffles could produce almost all of these compounds on their own [8].

None of the sulphur based compounds was found in the vegetative (mycelia) but a total of eight compounds were discovered compared to 200 in the fruiting body [12]. Fruiting bodies produce their own sulphur containing compounds. The nature of reactions that lead to volatile compounds production is as detailed in Fig 2.1.1:

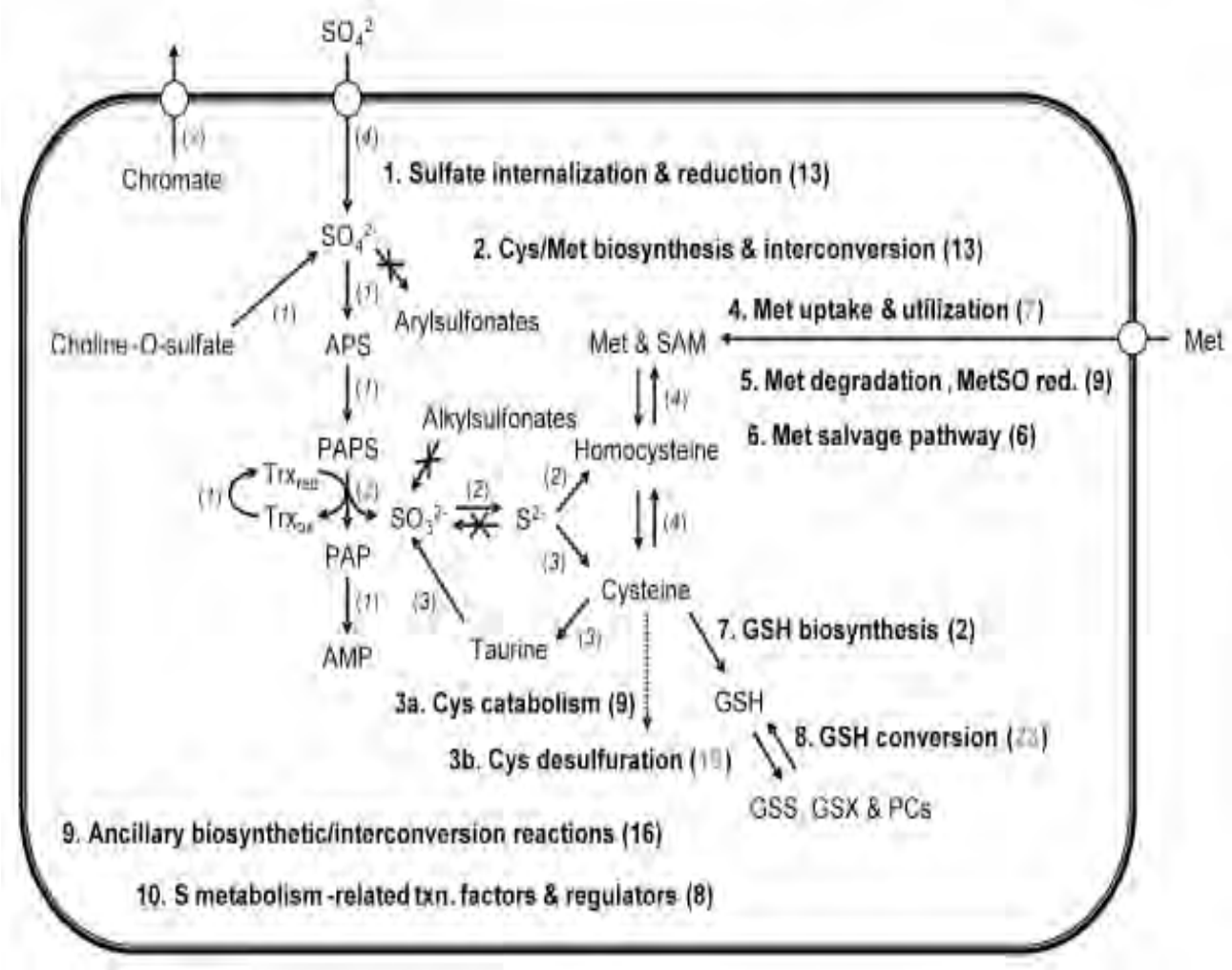


Fig. 2.1.2 Outline of sulphur metabolism and of the corresponding genes and pathways in *T. Melanosporum* [8].

There are ten known sulphur metabolism related sub pathways [8] taking place in the truffle fruiting body. The three main pathways exhibiting the strongest fruiting body bias are; "*sulphate internalization & reduction", Cyst/Met biosynthesis & interconversion" and methionine uptake and utilization".* A series of enzymatic reactions responsible for some of

the volatile compound production including; phytochelatin synthase, three putative dipeptidyl aminopeptidases and chromate efflux transporter and putative sulfate transporter have been reported [8].

There are many other reactions taking place within the fruiting bodies (Fig 2.1.3) including enzymes involved in S-amino acid biosynthesis/interconversion like methionine formation and recycling, cysteine/homocysteine interconversion (cystathionine γ-lyase and cystathionin synthases.

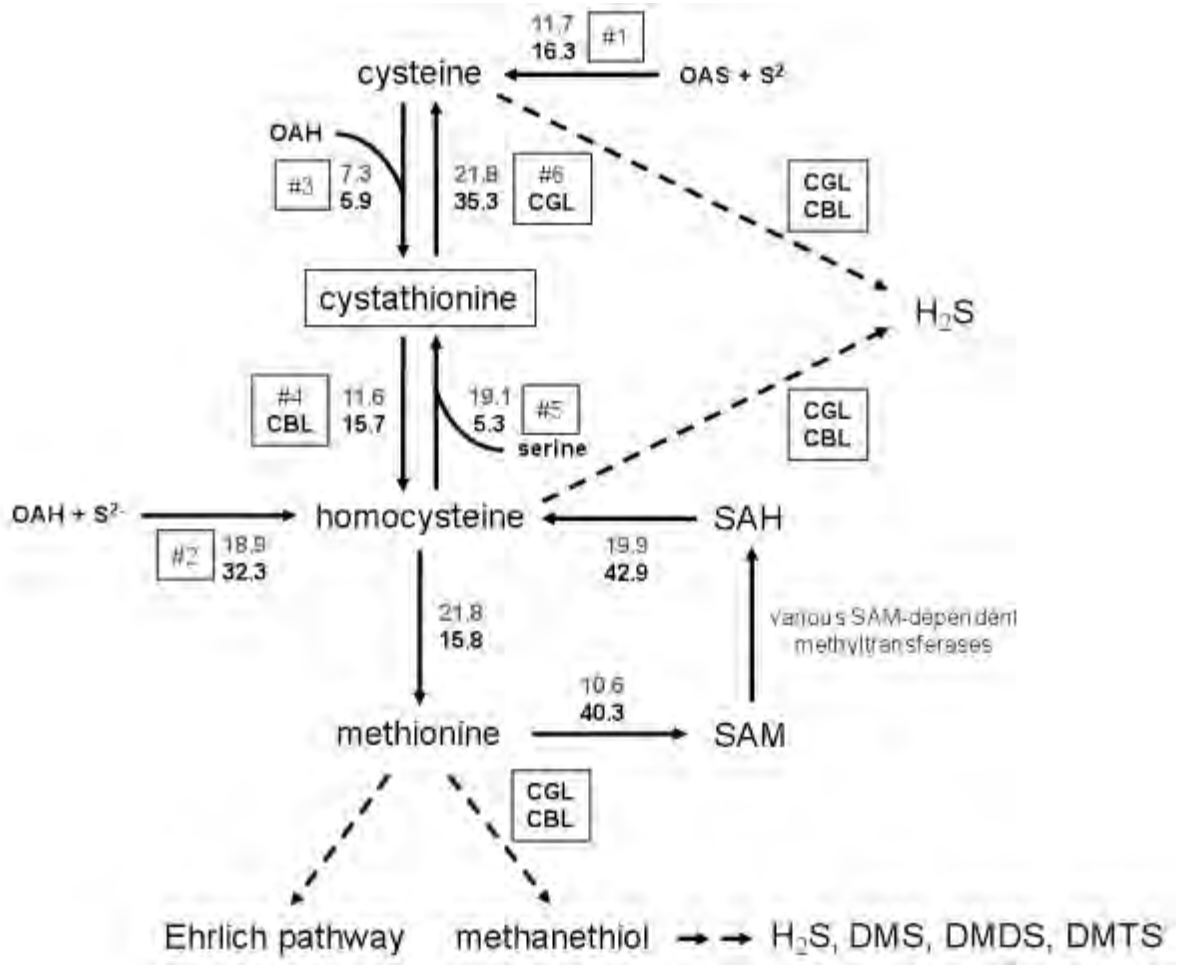


Fig. 2.1.3 Metabolic map of the Cys/Met biosynthesis & interconversion pathway and mRNA expression levels of the corresponding enzymes [8].

Expression levels are the mean of filtered and normalized hybridization signals derived from multireplicate experiments: expression values for free-living mycelia (FLM) and fruiting bodies (FB) are shown in *green* and *black*, respectively. Of note: i) the preference for homocysteine (rxn. #2) vs cysteine (rxn. #1) biosynthesis in FB; and ii) the disproportionately high expression levels of cystathionine γ-lyase (CGL, #5) and cystathionine β-lyase (CBL,

#4) in FBs compared to those of the preceding cystathionine synthase enzymes (#3 and #5), both of which are less expressed (3 to 6 fold) in FBs than in FLM. Alternative reactions supported by CGL and CBL homologs from lactic bacteria, potentially relevant for S-VOC formation in *T. melanosporum*, are indicated by dashed arrows. OAS, O-acetylserine; OAH, O-acetylhomoserine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; DMS, dimethylsulfide; DMDS, dimethyl-disulfide; DMTS, dimethyl-trisulfide [8].

Several microorganisms are known to act on various S-containing substrates besides cystathionine and these include cysteine/homocysteine desulfhydrylation and H_2S production and the dethiomethylation of methionine to methanethiol which then spontaneously decomposes to H_2S, dimethyldisulfide and other methyl sulfides which are known constituents of truffle flavor compounds. Other flavor related pathways originating from methionine are centered on 4-methylthio-2-oxobutanoic acid which can be degraded chemically or enzymetically to methanethiol and sulfides, or be converted enzymetically into 3-(methylthio) propanal and 3-(methylthio) propanol or the corresponding alcohols/acids through the Ehrlich pathway.

The technique employed for the extraction of volatile compounds in truffles was HS-SPME. A comparison of SPME and other techniques used in the analysis of VOCs is discussed in the next section.

2.2 Analytical techniques for VOCs

Extraction of the analytes from a complex matrix is a great challenge [11]. In order to improve accuracy in the measurement of VOCs, several approaches can be used such as:

- Head space techniques
- Static headspace extraction (SHE)
- Multiple headspace extraction (MHE)
- Dynamic headspace extraction (purge and trap)
- Liquid-liquid extraction with large volume injection
- Membrane extraction
- Solid-phase micro-extraction (SPME)

The choice of technique depends entirely on the needs of the analyst which essentially covers all or some of the following; sample matrix, whether information required is quantitative or qualitative, required sensitivity, automation and budget [11].

2.2.1 Headspace techniques

Figure 2.2.1.1 depicts stages in the development of headspace (HS) sampling and analysis in the 1960's [47].

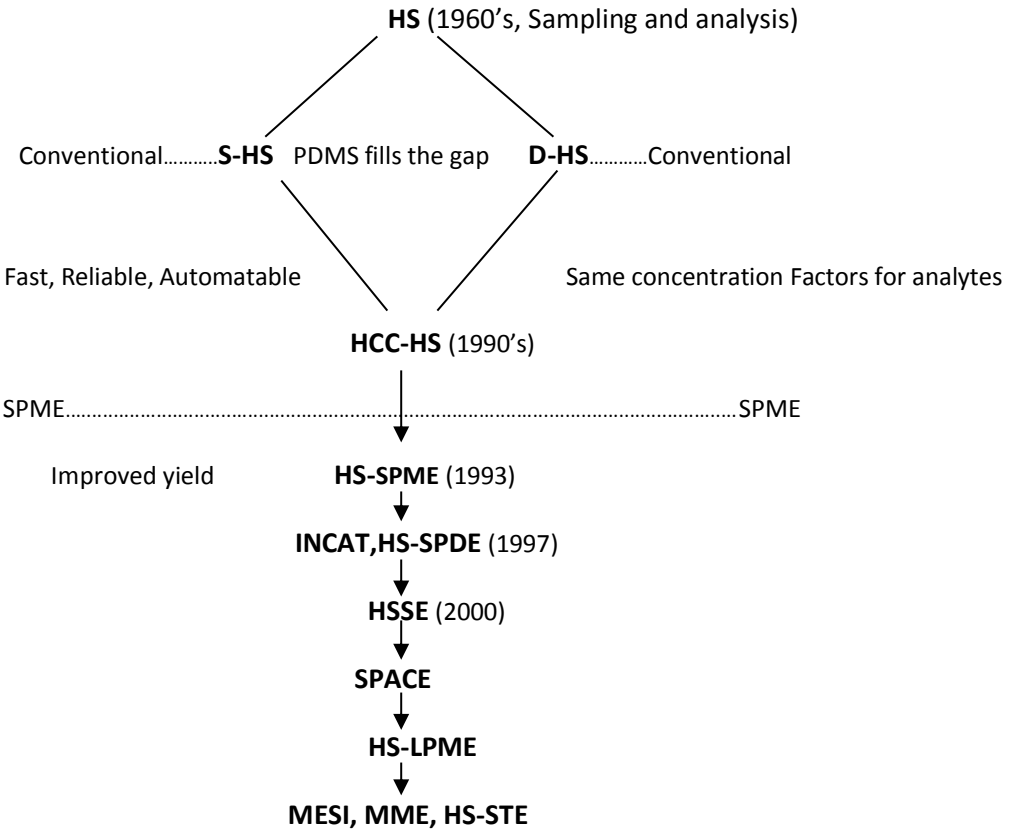


Fig. 2.2.1.1 Summary of the development of headspace techniques where; HS is headspace, S-HS is static headspace, D-HS is dynamic headspace, HCC-HS is high concentration capacity headspace, SPME is solid phase microextraction, HS-SPME is headspace solid phase microextraction, INCAT,HS-SPDE is inside needle capillary adsorption trap, headspace solid phase dynamic extraction, HSSE is high capacity headspace sorptive extraction, SPACE is solid phase aroma concentrate extraction, HS-LPME is headspace liquid phase microextraction, MESI is membrane extraction sorbent interface, MME is membrane microextraction and HS-STE is headspace sorptive tape extraction.

Literature has stated that the systems used were static headspace (S-HS) and dynamic headspace (D-HS) [47]. High concentration capacity headspace (HCC-HS) was then introduced around the same time with SPME in 1990's. HS-SPME was introduced in 1993 and improved analyte yield. HS-SPME is the technique used in this study with headspace sampling to minimize matrix effect.

The SPME system has since been exposed to further developments. 1997 saw the introduction of inside needle capillary adsorption trap, headspace solid phase dynamic extraction (INCAT, HS-SPDE) [47]. The high capacity headspace sorptive extraction (HSSE) was developed in 2000 and subsequently followed by solid phase aroma concentrate extraction (SPACE), headspace liquid phase microextraction (HS-LPME), membrane extraction sorbent interface (MESI), and membrane microextraction (MME) which are all large surface area high concentration capacity headspace (HCC-HS) sampling techniques. The different head space techniques are discussed in detail under the following sections.

2.2.1.1 Static headspace extraction (SHE)

This is also known as *equilibrium headspace extraction*. It is often referred to as simply *headspace*. Static headspace is common for both qualitative and quantitative analysis of volatile organic compounds [11]. It is applicable to a variety of matrices.

This technique of extraction is very simple. The sample (solid or liquid) is placed in a head space auto sampler (HSAS) vial and the volatile analytes diffuse into the headspace of the vial. The concentration of the analytes in the headspace of the vial then reaches equilibrium with the concentration of the analytes in the sample matrix. A fraction of the headspace of the vial is then introduced into a gas chromatograph for analysis either manually or by automation. The system uses the concept of balanced pressure sampling for the introduction of analytes into the GC. As the vial is directly connected to the GC through a heated transfer line, a pressure drop ensures transfer of analytes into the GC column. SHE technology has been in the market for more than 30 years [13] and it has proven to be reliable with an ease of sample preparation being an added advantage.

2.2.1.2 Multiple headspace extraction (MHE)

The technique is used to measure the total peak area of an analyte. This allows for the determination of the total amount of analyte in the sample. The technique is backed by a mathematical model [14-15]. The mathematical concepts of this technique are detailed by Kolb and Ettre [16].

The model [15] states that if a gas extraction is carried out in a continuous mode, the concentration of the analyte in the purge gas will first be high and then decrease with time. The decrease is exponential hence the concentration (c) versus time (t) relationship can be described by an equation of the first order as in equation 1;

$$-dc/dt = q.\ c \qquad (1)$$

where; q is a constant, $-dc$ is the change in concentration and dt is change in time and c is the final concentration.

In summary the model states that the total amount of a volatile analyte (Eq. 2 and 3) present in the sample can be calculated from the sum of all partial peak areas, obtained in a series of chromatograms [15] hence expressed in equation 2;

$$A_i = A_1+A_2+...An \qquad (2)$$

where; A_i is the total amount of analyte present and the terms on the right ($A_1+A_2+...An$) represent different peak areas of the analyte for n number of extractions as in equation 3;

$$A_i = A_1\,[1+e^{-q'}+e^{-2q'}+...+e^{-(n-1)\,q'}] \qquad (3)$$

Equation 3 is a geometric with the quotient $e^{-q'}$, A_1 being the peak for the analyte of interest. The constant q is replaced by q' to account for the additional parameters brought by the instrument during automation and n is the number of extractions performed.

The technique eliminates matrix effect concerns which are common in solid samples because it examines the whole analyte amount [11]. Consecutive tests are performed on the same sample vial while the partition coefficient K remains constant. The declining peak areas are monitored until finally they fall to zero after all the analyte has been exhausted. In the laboratory a series of extractions are followed by linear regression analysis to evaluate the total amount of analyte present in the sample.

2.2.1.3 Dynamic headspace extraction (Purge and trap)

Purge and trap dynamic headspace is often preferred over other extraction techniques for the analysis of analytes at trace levels [11]. This technique relies on the volatility of the analytes for extraction from the matrix. In this case, the volatiles are not accumulated through reaching equilibrium between gas phase and matrix, instead a continuous flow of a gas (which creates a concentration gradient) carries the analytes straight from the sample into the analytical instrument. The technique is applicable to both liquid and solid samples from a wide range of complex matrices. It is applicable to environmental samples [17-19], biological [19-20] industrial, pharmaceutical and agricultural samples. EPA approved standard methods apply this technique [21-23]. Quantitation is easy with external standards [11].

2.2.2 Liquid extraction with large volume injection

The technique involves the use of large volume liquid injections of up to 150 µL. Volumes of 1 to 2 ml can also be injected into the GC within a short time period [11]. Current technology has combined robotic sampling with low extraction volumes allowing for trace analysis. COMBI-PAL sampling technique makes this type of analysis possible. Large volume injections use two methods namely solvent vapour exit (SVE) and programmed temperature vaporization (PTV). SVE is based on the classical on-column inlet and PTV on split/splitless inlet. The former is best used for clean samples like drinking water [11].

2.2.3 Membrane extraction

Membrane extraction has been applied to a range of analytes from different matrices [11]. It can be coupled to an instrument for continuous online analysis like MS [24-35] or GC [36-45]. In membrane extraction, the sample contacts just one side of the membrane (the feed) while the analytes pass through to the other (permeate) side. The membrane can easily be coupled to a GC for an online injection / analysis. In the extraction of volatile compounds, the application is referred to as *membrane pervaporation*. This refers to the selective evaporation of liquid molecules. The analytes that have passed through are driven by a gas

into a trap for preconcentration, before the trap is heated up to desorb them into the GC. The limit of detection for this technique is in the ppt to ppb range [11].

2.2.4 Solid phase micro-extraction (SPME)

SPME was first introduced by Arthur and Pawliszyn in 1990 [17]. The technique added improvement to already existing headspace sampling which was in use since the 1960s [47]. It brought upon an improvement of the headspace sampling technology with notable advantages over the conventional ways, mainly elimination of the matrix effect [11]. This has made SPME applicable to a wide range of complex material as in; environmental, food, biological and pharmaceutical products [4].

The development of SPME has seen the concept of miniaturizing extractions using solid phase sorbents evolving into a number of different approaches. The extracting phases of this technique are not always solids [46]. Polydimethylsiloxane (PDMS) is a single component liquid absorbent.

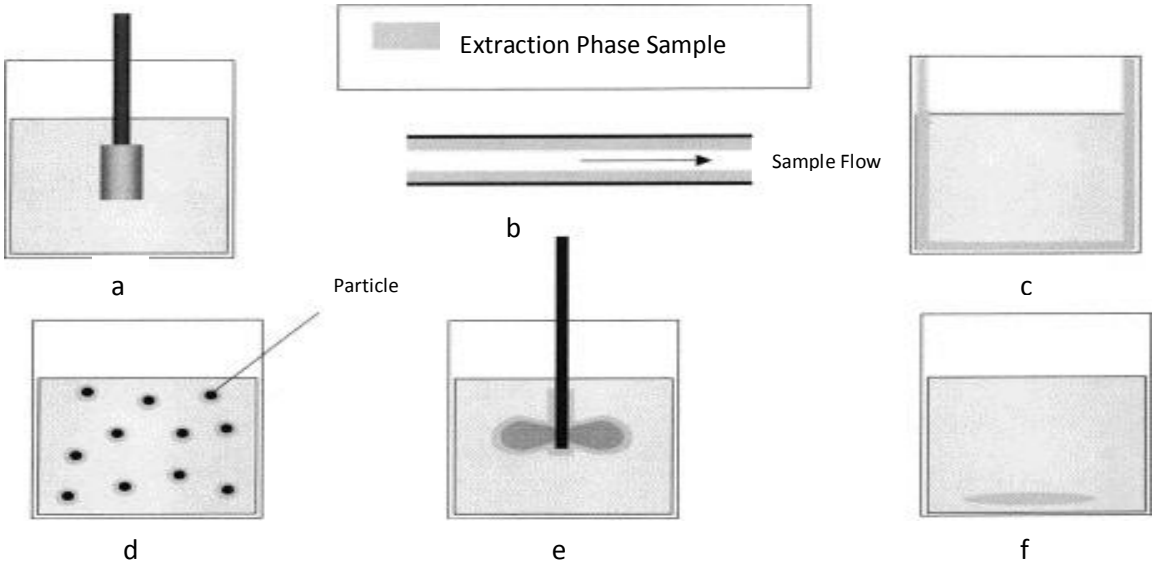


Fig. 2.2.4.1 Configurations of solid-phase microextraction: (a) fiber, (b) tube, (c) vessel walls,(d) suspended particles, (e) stirrer, and (f) disk/membrane. [11]

All configurations shown in Fig 2.2.4.1 are considered as variations on the basic SPME theme. The most common SPME application is the use of sorption by exposing the sample to

a thin layer of sorbent coated on the outer surface of fibers (Fig 2.2.4.1 a) or on the interior of a capillary tube (Fig 2.2.4.1 b). Fibers were used in this research work. There is a common extraction mechanism that defines why methods in figure 2.2.4.1 have been grouped together. SPME cannot attain exhaustive extraction [11]. In SPME, samples are analysed upon reaching equilibrium or after a certain predetermined time. SPME has got a big advantage of being a solventless procedure ruling out solvent disposal [49,50]. It is also very simple to use because the concept is based only on two stages; sorption and desorption [51]. High concentration capacity and selectivity are some of the main advantages of SPME. Compared to the conventional SPE which does exhaustive extracts (> 90%) of the analyte with only 1 to 2% of the sample injected into the analytical instrument, the non exhaustive SPME only extracts 2 to 20% of the analyte then all the sample is injected into the instrument [49, 51, 52]. SPME technique can allow for use of very small sample sizes for extraction. Since its non exhaustive, the technique could also be used in living systems without shifting their chemical equilibria [46]. The biggest challenge of SPME is the matrix effects, changes in the complex sample matrix would normally affect the distribution constant value [49, 52]. Samples analysed by SPME have to be relatively clean though it is applicable to complex environmental matrices. Dirty samples pose a challenge.

The polymer coating on the fiber can be liquid or solid or a mixture of the two. SPME system works by equilibration (matrix/headspace and headspace/fiber). A linear relationship is observed between the number of moles of the analyte adsorbed on the fiber and the concentration of the analyte in the aqueous phase [46]. The equation of the relationship is thus expressed as in equation 4;

$$n = \frac{KfsVfVsCo}{KfsVf+Vs} \qquad (4)$$

where;

n = number of moles of the analyte.
K_{fs} = fiber coating / sample matrix distribution coefficient.
V_f = volume of the fiber coating.
V_s = sample volume.
C_o = initial concentration of analyte

The advantages of SPME are elimination of the matrix effect, simplicity of operation, use of no solvents, its fast and highly selective. It however has limitations of poor reproducibility, intolerance for organic solvents and there is a limited range of fibers available commercially and it is expensive [60]. Different fiber coating descriptions and applications are covered under section 2.2.7 which includes: polydimethylsiloxane (PDMS), divinylbenzene (DVB), polyacrylate (PA), carboxen (CAR) and carbowax (CW). Fibers come in different coating combinations and coating phase thicknesses for an increased field of application. Different configurations of SPME (Fig 2.2.4.1) rely on the geometry of the extraction phase like; coated fibers, vessels, stir bars, disks and the inside tube coatings. The coated fiber was selected because of its convenience and ease of use. Analytes are directly desorbed into the GC injection port.

In SPME, the fiber is exposed to the sample matrix or the headspace. The polymer coating is therefore exposed until equilibrium is attained between the analyte adsorbed on the fiber coating and the analyte in the sample matrix. The concentration of analyte adsorbed on the fiber, due to equilibrium reached, is proportional to the initial concentration of the analyte in the matrix [60].

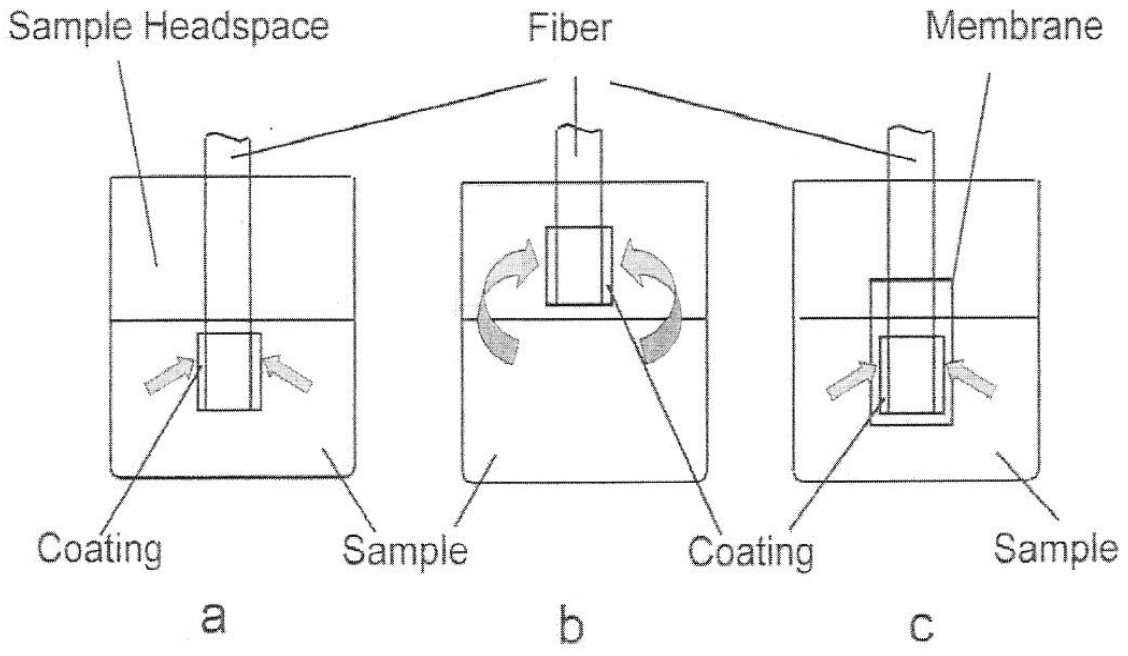


Fig. 2.2.4.2 SPME modes (a) direct immersion (b) headspace SPME (c) membrane protected SPME [11]

Fig 2.2.4.2 shows three modes of SPME. Direct immersion involves the coated fiber being immersed directly in the sample matrix for the analytes to interact directly with the fiber coating. Fast transfer of analytes to the fiber coating may be enhanced by agitation. The common agitation techniques are ultrasonication and use of a stirring bar, usually for aqueous matrix.

There is no need for agitation in the headspace mode; the gas phase easily interacts with the fiber coating material [11]. In this arrangement the fiber is well protected from corrosive chemicals within the sample matrix and the conditions of the sample can be altered without interfering with the fiber [61].

Headspace SPME is the best choice for volatile analysis because volatile analytes tend to be more concentrated in the headspace than in the sample (liquid) matrix [11]. Diffusion rates are higher in the gaseous phase by up to 4 orders of magnitude as compared to the liquid medium and volatiles equilibrate faster in headspace. The membrane in SPME serves to protect the fiber against rough conditions in dirty samples. Specific membranes can help improve fiber selectivity [40].

2.2.5 Sorbents used in SPME

SPME sorbents are commonly immobilized by coating them onto fused silica fibers [53]. Coating is either done on the outside of the fused silica fibers or on the inside of a capillary tube. The coating phases are normally not chemically bonded to the fused silica. However, for a PDMS of 7 μm thickness the coating is bonded to the silica fiber. Cross linking is used to ensure stability of the other coatings in organic matrix [54]. Carboxen in the context of SPME refers to “Carboxen 1006”, a trade name for porous synthetic carbons which have a high distribution of micro, meso and macropores that promote rapid desorption.

There are several fiber coating phases which differ in polarities, absorbent properties and porosities. This section shall address all available choices with emphasis on fibers selected for this study.

2.2.6 Polydimethylsiloxane (PDMS)

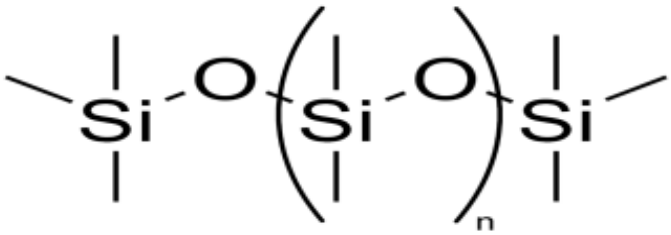


Fig. 2.2.6.1 Chemical structure of polydimethylsiloxane [4]

Polydimethylsiloxane (PDMS) is nonpolar. It is a single component liquid absorbent. PDMS phase coatings come in different film thicknesses of 7, 30, and 100 µm [54]. PDMS is compatible with GC and HPLC applications. 100 µm PDMS coating is widely used for volatile compounds by headspace. PDMS 30 µm is the intermediate level coating in this series and it finds its applications in the analysis of nonpolar semivolatile organic compounds. PDMS 7 µm, which is the thinnest film of this range is used for the analysis of nonpolar, high molecular weight volatile compounds. PDMS fibers operate optimumly at a pH range of 4 to 10 [54]. They also have optimum molecular weight range for analytes.

2.2.7 Polyacrylate (PA)

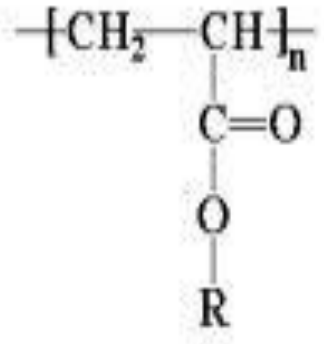


Fig. 2.2.7.1 Chemical structure of polyacrylate [4]

The fiber which was used for polar analytes in this study was Polyacrylate (PA), which is a single-component polar absorbent coating with 85 µm film thickness. This sorbent is also

compatible with both GC and HPLC applications (extraction) for semi volatile compounds [11].

2.3 Multiple-Component Phases

Besides the two types of sorbent coatings stated, there are other fiber coating phases which are multiple phased (Table 2.2.5.1). These are available in different forms such as: porous, adsorbent and blended particle phases.

2.3.1 Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS)

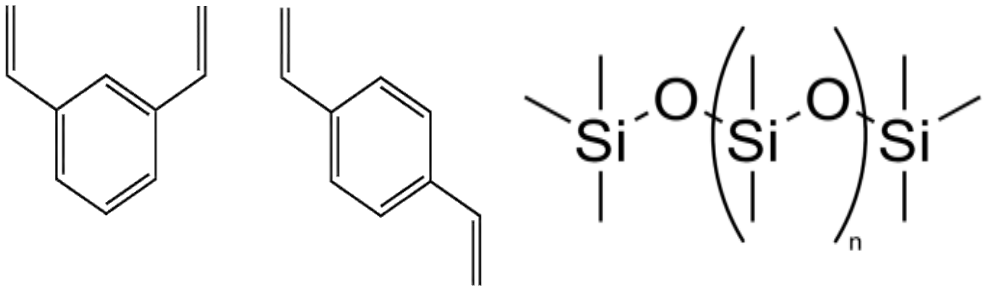


Fig. 2.3.1.1 Chemical structure of DVB/CAR/PDMS [4]

The adsorbent fiber coating used in this work consisted of divinylbenzene (DVB) and Carboxen particles attached to PDMS (DVB/CAR/PDMS 50 /30 μm). The multiple component fiber has a bipolar phase (DVB/PDMS- 50 μm on CAR/PDMS 30 μm). Its advantage is that the analyte molecular range to be captured by the fiber is expanded [56]. The meso and macropores of the layer of DVB (outer part of the coating) trap the larger molecular size analytes. Micropores of the inner Carboxen layer trap smaller analytes which passed untrapped by the DVB layer. This double layer finds common application in aroma/odor compounds, volatile and semi volatile flavor compounds in GC analysis [57]. Due to high affinity of small amines to DVB, the combination of DVB over Carboxen makes it the best choice for the extraction of isopropylamine. It has to be noted that for

DVB/CAR/PDMS 50/30 µm phase, instead of using the non polar PDMS, a moderately polar phase Carbowax (CW) can be used to suspend DVB and Carboxen particles [54].

Other fiber coating phases discussed in the next sections do not form part of the scope of this study but are an essential part of this subject. They are all multiple-component phases which extensively exploit the adsorbent process of SPME.

2.3.2 Polydimethylsiloxane-Divinylbenzene (PDMS-DVB)

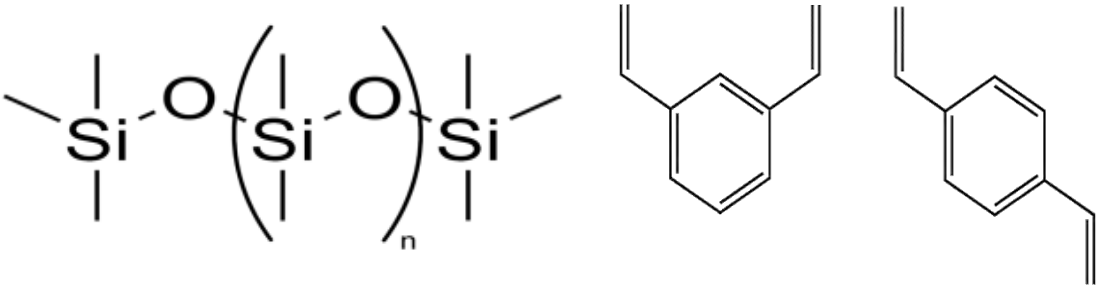


Fig. 2.3.2.1 Chemical structure of PDMS-DVB [4]

PDMS-DVB 65 µm (DVB suspended on PDMS) is normally used for the extraction of volatile, amine, or nitroaromatic analytes (GC analysis). A 60 µm film thickness of this phase is used for HPLC analysis of amines and polar compounds [55].

2.3.3 Carbowax-Divinylbenzene (CW-DVB)

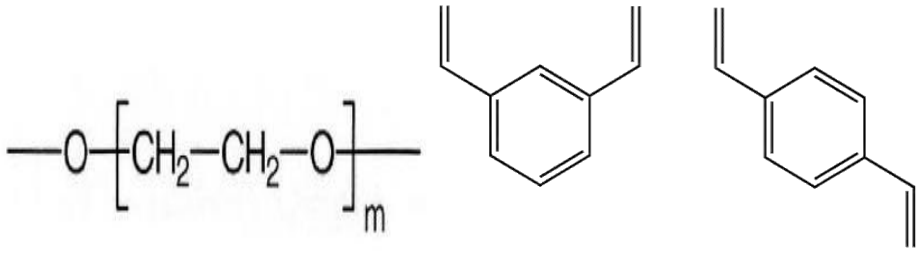


Fig. 2.3.3.1 Chemical structure of CW-DVB [4]

CW-DVB (DVB suspended on Carbowax) comes in 65 µm or 70 µm film thickness. It is polar sorbent and applied on GC analysis of alcohols and polar compounds [53, 55].

2.3.4 Carboxen/Polydimethylsiloxane (CAR/PDMS)

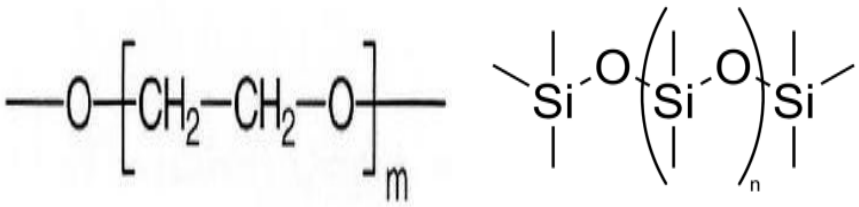


Fig. 2.3.4.1 Chemical structure of CAR-PDMS [4]

The bipolar Carboxen/PDMS (Carboxen suspended on PDMS phase) comes in 75 µm or 85 µm film thickness. This phase finds its use in the GC analysis of gases and low molecular weight compounds with the latter being best for analysis of molecular weights < 90 [55].

2.3.5 Carbowax/Templated Resin (CW/TPR)

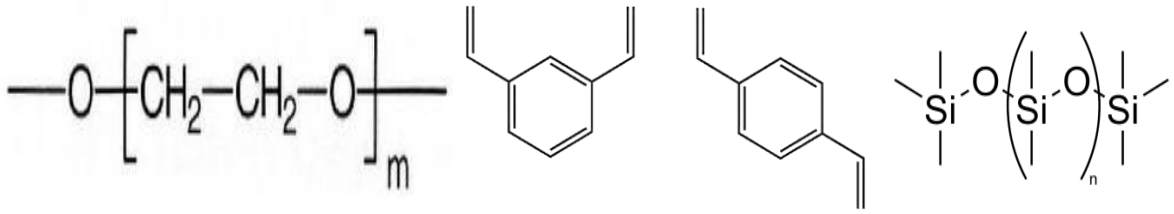


Fig. 2.3.5.1 Chemical structure of CW-TPR [4]

CW/TPR 50 µm (Carbowax / Templated resin) is used for HPLC analysis of surfactants. The templated resin is a hollow, spherical DVB which has been made by coating a silica template with DVB [55].

In summary the choice of sorbent is dictated to a large extent by analyte size (has got a direct link with the diffusion coefficient of the analyte in the sample matrix and in the sorbent), concentration levels and limits of detection [55]. Different SPME fiber coatings are commercially available depending on the required application (See Table 2.2.5.1).

Table 2.2.5.1 SPME fiber selection guide [11]

Analyte Class	Fiber Type	Linear Range
Acids (C2-C8)	Carboxen-PDMS	10 ppb-1 ppm
Acids (C2-C15)	CW-DVB	50 ppb-50 ppm
Alcohols (C1-C8)	Carboxen-PDMS	10 ppb-1 ppm
Alcohols (C1-C18)	CW-DVB	50 ppb- 75 ppm
	Polyacrylate	100 ppb-100 ppm
Aldehydes (C2-C8)	Carboxen-PDMS	1 ppb-500 ppb
Aldehydes (C3-C14)	100 m PDMS	50 ppb-50 ppm
Amines	PDMS-DVB	50 ppb-50 ppm
Amphetamines	100 m PDMS	100 ppb-100 ppm
	PDMS-DVB	50 ppb – 50 ppm
Aromatic amines	PDMS-DVB	5 ppb-1 ppm
Barbiturates	PDMS-DVB	500 ppb- 100 ppm
Benzidines	CW-DVB	5 ppb-500 ppb
Benzodiazepines	PDMS-DVB	100 ppb-50 ppm
Esters (C3-C15)	100 m PDMS	5 ppb-10 ppm
Esters (C6-C18)	30 m PDMS	5 ppb-1 ppm
Esters (C12-C30)	7 m PDMS	5 ppb-1 ppm
Ethers (C4-C12)	Carboxen- PDMS	1ppb-500 ppb
Explosives (nitroaromatics)	PDMS-DVB	1 ppb-1 ppm
Hydrocarbons (C2-C10)	Carboxen-PDMS	10 ppb-10 ppm
Hydrocarbons (C5-C20)	100 m PDMS	500 ppt-1 ppb
Hydrocarbons (C10-C30)	30 m PDMS	100 ppt-500 ppb
Hydrocarbons (C20-C40)	7 m PDMS	5 ppb-500 ppb
Ketones (C3-C9)	Carboxen-PDMS	5 ppb-1 ppm
Ketones (C5-C12)	100 m PDMS	5 ppb-10 ppm
Nitrosamines	PDMS-DVB	1 ppb-200 ppb
PAHs	100 m PDMS	500 ppt-1 ppm
	30 m PDMS	100 ppt-500 ppb
	7 m PDMS	500 ppt-500 ppb

2.4 Supercritical fluid extraction (SFE)

Supercritical fluids were discovered by Baron Cagniard de la Tour in 1822 [58]. A supercritical fluid refers to a substance above its critical temperature and pressure as shown in Fig 2.4.2. A gas does not liquefy under increasing pressure beyond the critical temperature. It is rather compressed into a supercritical fluid. The critical point differs from one substance to another. Due to their unique properties, supercritical fluids have solvating power, diffusivity better than those of liquids, low viscosity and negligible surface tension like gas.

Between 1964 and 1976, Zosdel patented methods on decaffeination of coffee; this started a major development in SFE [59]. The technique has found many industrial applications since then. Supercritical fluid chromatography was introduced in 1981 [59] for analytical purposes. The work presented in this study discusses extraction of volatile organic compounds by HS-SPME and supercritical carbon dioxide. This is based on the fact that with rapid mass transfer in the supercritical phase and better ability to penetrate the sample matrix, extraction is fast in SFE together with high extraction efficiency. SFE makes use of the special properties of supercritical fluids to facilitate the extraction of organics from solid samples. SFE can either be applied on-line or off-line. The technique was used off-line for this work; a stand-alone extraction method independent of the analytical technique was used.

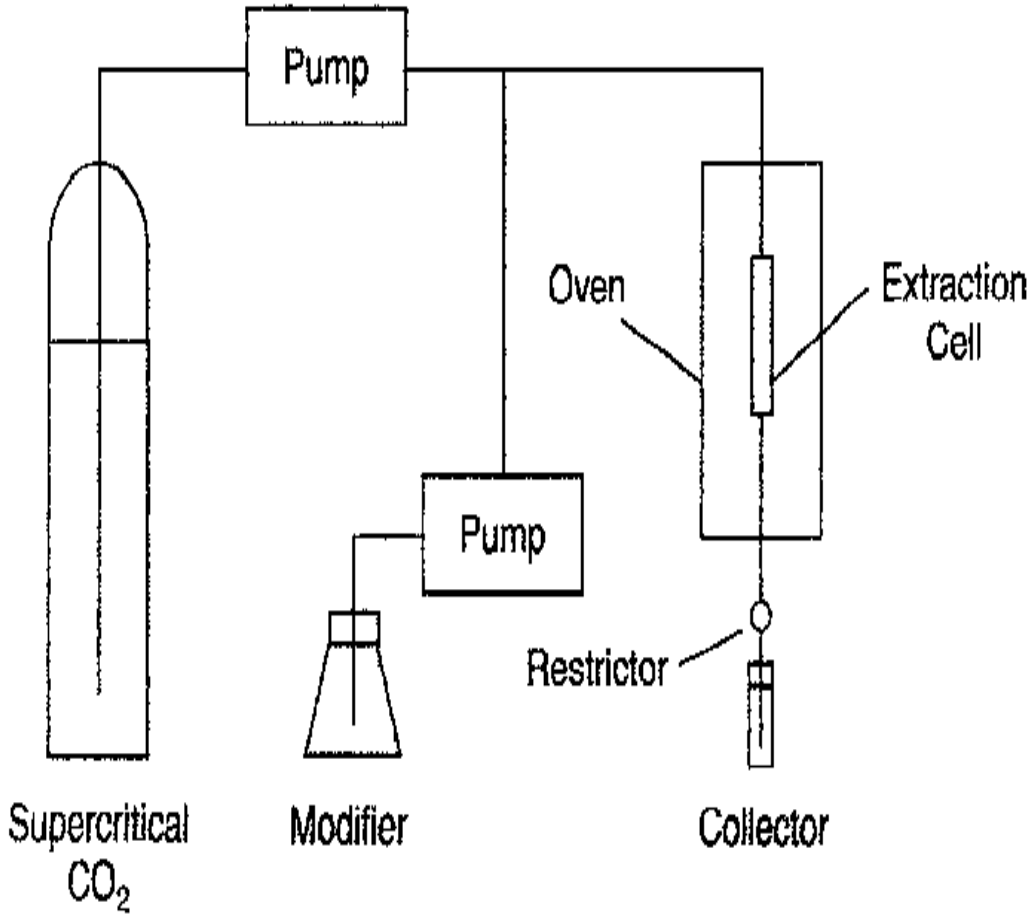


Fig. 2.4.1 Schematic diagram of an off-line SFE system [38]

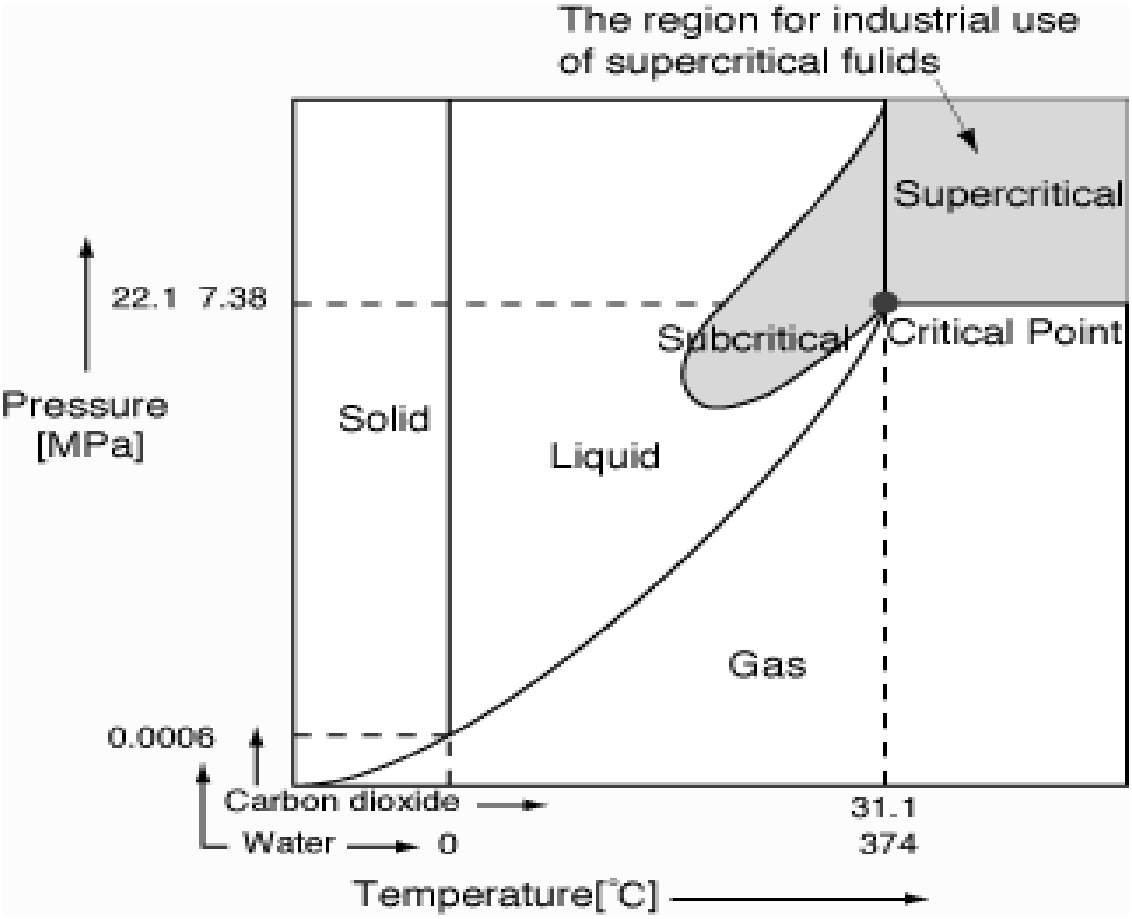


Fig. 2.4.2 Phase diagram of pure carbon dioxide [9]

The use of liquid CO_2 is popular in SFE as it is a green solvent. CO_2 has a low supercritical temperature of 31 °C and pressure of 73 atmospheres. It has high purity levels and it is non toxic. This solvent is nonpolar, without dipole moment hence a good solvent for extraction of nonpolar and moderately polar compounds just as the study required. CO_2 has a disadvantage of a poor solvating power for polar solutes, the solutes which bound strongly to the matrix make CO_2 solvent strength inadequate to break the solute-matrix bond.

Table 2.4.1 Critical parameters of select substances [11]

Substance	Critical Temperature (°C)	Critical Pressure (atm)	Critical Density (10^3 kg/m^3)
CO_2	31.3	72.9	0.47
N_2O	36.5	72.5	0.45
SF_6	45.5	37.1	0.74
NH_3	132.5	112.5	0.24
H_2O	374	227	0.34
n-C_4H_{10}	152	37.5	0.23
n-C_5H_{12}	197	33.3	0.23
Xe	16.6	58.4	1.10
CCl_2F_2	112	40.7	0.56
CHF_3	25.9	46.9	0.52

SFE employs fluids in their supercritical states for the extraction of solid samples. Supercritical fluids behave like gases although they have the density of liquids and as a result, they have a high diffusivity, low viscosity, good penetration capability and adjustable density [63]. SFE offers an advantage of tenability. The change in density makes SFE applicable to a higher range of materials. It has shorter extraction times, lower solvent consumption and suitability for thermally labile compounds. This technique can be used at reduced working temperature. Supercritical carbon dioxide has been applied to lipid and lyophilic components extraction [64]. It has proven to perform better than other conventional solvents and high extraction rates were observed at low temperatures while thermo labile components are preserved in the process [65]. In SFE, a solid or semi-solid sample is placed in a pressure vessel and extracted with a re-circulated stream of supercritical fluid which is well mixed with the sample matrix. This allows analytes to transfer to the fluid. The extract is collected into a vial or cartridge (Fig 2.4.1).

SFE involves three main steps;

1) Initial partitioning of the analyte from the matrix into the supercritical fluid
2) Elution of analyte from the extraction cell
3) Collection of the analytes.

The first step depends on factors such as diffusion of the analyte between the matrix active sites and the ability of the supercritical fluid to initially displace the analyte from these sites. This initial desorption step is often the rate determining step in the SFE of most complex environmental samples [66]. All three steps contribute to the overall extraction efficiency [67].

The elution of the analyte from the extraction cell depends on the amount of fluid flow in relation to sample size as well as the solubility of the analyte in the supercritical fluid. The collection relies on the restrictor type on the SFE instrument and the trapping arrangement for the extract [68].

The density of a supercritical fluid is defined by temperature and pressure settings. At constant pressure, the density of CO_2 decreases when the temperature is increased. An increase in pressure at a given temperature results in an increase in fluid density.

2.5 Pressurized hot water extraction (PHWE)

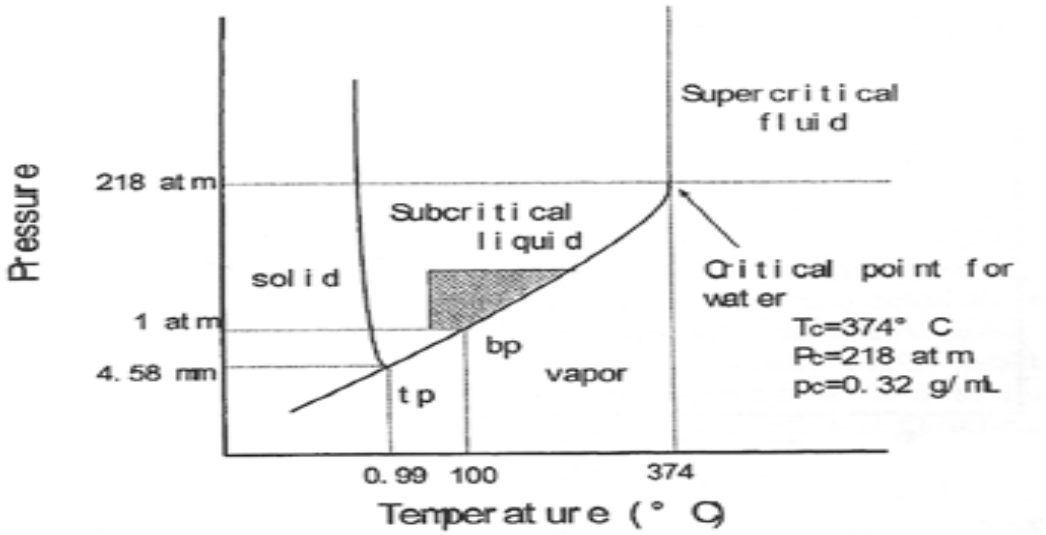


Fig. 2.4.3 Phase diagram of water [9]

PHWE (Water at elevated temperature and pressure) has been applied before in food matrices as an effective analytical technique. The advantages of this solvent are that; It is tunable, fast and efficient due to high diffusion coefficient, it does not pose degradation problems and it is a sustainable solvent. Pressurized hot water is highly selective due to the ability to regulate its dielectric constant and it has been used for the extraction of pesticides, essential oil, anthocyanins, lipids/oils, carbohydrates, proteins and in hydrocarbon separation [64]. The only challenge can be removal of water from the sample after extraction. The thesis covers work done on the application of PHWE (static and dynamic modes) for the extraction of bioactive compounds of truffles and their subsequent analysis. When optimized, this technique is mostly applied in four critical areas of (1) Processing scaling up, (2) Subcritical water chromatography, (3) Analyte solubility predictions and (4) Increase of analyte flux rates due to mass transfer [64].

This work looks at pressurized hot water extraction (PHWE) performed using two different sets of equipment one in the static mode and the other in a dynamic mode.

A pressurized static batch reactor (Fig 3.4.4.1) with a cell volume of 100 mL (Mini Reactor, Autoclave Engineers, Erie, PA, USA). The reactor was comprised of; a heating unit, stirrer, inlet N_2 gas, and a filter for both sample inlet and outlet valves

Dynamic hot water extraction was performed using an in-house built system made of an adopted old GC oven (Fig 3.4.5.1) comprising; HPLC pump, stainless steel pipes, sample cells (with inlet and outlet filters), pressure regulator and sample collection outlet.

2.6 Identification techniques

Modern analytical techniques were employed in this study to elucidate the nature, structure and composition of the volatile organic compounds of truffles. The techniques used are found in most comprehensive chemistry research centres, hence they form the backbone of chemical analysis.

2.6.1 Infra-red spectroscopy

Infra-red radiation has a lower frequency range than visible light. A range of infra-red frequencies are typically absorbed by molecular vibrations The frequency absorbed will depend on the compound, its structure and the type of vibration it undergoes. The main components of an infra-red spectrophotometer are: an infra-red radiation source, a monochromator to scan through a range of frequencies, front-surfaced mirrors, a sample chamber and a detector. The region usually scanned is called mid infra-red. This is where the majority of absorption occurs. It covers 200 -4000 cm^{-1}. The most common type of vibrations that cause absorption of infra-red radiation are bending and stretching vibrations. A typical infra-red spectrum of an organic compound has three distinct regions. The first region is 4000 – 2500 cm^{-1}, the second being 2500 – 1500 cm^{-1} and the third region being 1500-400 cm^{-1}. The first region usually covers bond stretching between H and atoms such as C and O. The second region, which is the centre region of the spectrum is occupied by stretching of double bonds (e.g. C=O, C=C, C=N). The carbonyl stretch at 1750 – 1700 cm^{-1} is found in this region. Bending peaks of N-H and absorption by aromatic rings are also found in this region. The third region is commonly referred to as the fingerprint region. This displays peaks due to stretching and bending. Compounds will display very characteristic traces in this region. Comprehensive tables and libraries containing characteristic infra-red absorption bands exist. These normally provide sufficient information to identify the presence of the main functional groups [72].

2.6.2 Mass spectrometry

Mass spectrometry measures comparative masses of chemical species. These are expressed in terms of mass to charge ratio denoted m/z. The mass spectrometer is typically composed of five main sections: Sample inlet, Ionisation chamber, Accelerator, Magnet and Detector. A single element passed through a mass spectrometer will generate a single peak. In that case, the relative mass will be determined with the relative abundance of 100 % where there is a single species. Isotopes can be analysed by mass spectrometry and their relative abundances expressed [72].

The ionisation process used by a mass spectrometer causes fragmentation of compounds. As a result a number of different species are generated. The mass spectra is analysed to identify compounds by firstly noting the molecular ion (The heaviest ion present which corresponds to the compound missing one electron).The second stage is to identify the base peak (The most abundant species with the highest relative intensity. The weights of the molecular peak and the base peak are noted. The structure is determined by studying the fragments presented in the mass spectrum in comparison with library search for suggested structures. Modern databases allow for cross referencing of newly recorded data with previously archived data.

In this work two techniques based on mass spectrometry were used. The LCMS studies involved the use of negative electrospray ionisation (ESI) as the ionisation mode for the fragmentation and positive identification of amino acids and fatty acids. ESI is known to be an excellent technique for the production of molecular ions from large polar molecules [73]. In ESI, an electrospray is produced when a potential difference is applied between the end of a capillary and a cylindrical electrode to a small flow of liquid sample from a capillary needle. The electrospray formed consists of highly charged liquid droplets which can either be positively or negatively charged depending on the sign of the voltage applied to the capillary. Solutions used in electrospray work are typically water / methanol mixtures containing the analyte. In this study PHWE were used.

GCMS work on volatile compounds utilised electron impact (EI) as the ionisation mode of choice. In EI, molecular ions produced from organic molecules can fragment either by loss of a radical or by loss of a molecule with all its electrons paired [73]. This happens as a result of electrons accelerating from the filament to an anode through a potential difference. The process produces enough energy to ionise organic molecules and also cause fragmentation.

This gives rise to a pattern of fragment ions which can help to characterize the compound. EI was used in this study to characterize volatile organic compound of truffles. Compound identities were confirmed using Saturn library software.

Chapter 3

3.0 Experimental

3.1 Overview

Truffles were characterised for morphology by SEM. Oil and water extracts were characterised for functional group components by FTIR. Pressurized hot water extraction (PHWE) was employed for the extraction of bio-active compounds (amino acids and fatty acids) and analysed by LCMS. Volatile compounds were extracted and pre-concentrated from Kgalagadi desert truffles (*kalaharituber*) by headspace, headspace solid phase microextraction (HS-SPME) and supercritical fluid extraction (SFE). Four different types of HS-SPME fibers (PDMS 100 µm, PDMS 7 µm, Polyacrylate 85 µm, CAR/DVB/PDMS 50/30 µm) were evaluated for suitability and selectivity. Subsequent analysis of volatile compounds was carried out employing a gas chromatograph coupled with mass spectrometric detection.

3.2 Chemicals and reagents

Gold, Hexamethyldisilazane, glutaraldehyde, sodium phosphate and ethanol were supplied by Merck Chemicals (Johannesburg, South Africa). Volatile organic compounds standard mixture (EPA 524 VOC Mix A; Benzene, Bromobenzene, Bromochloromethane, Bromodichloromethane, Bromoform, Butylbenzene, *sec*-Butylbenzene, *tert*-Butylbenzene, Carbon tetrachloride, Chlorobenzene, Chloroform, 2-Chlorotoluene, 4-Chlorotoluene, Dibromochloromethane, 1,2-Dibromo-3-chloropropane, 1,2-Dibromoethane, Dibromomethane, 1,2-Dichlorobenzene, 1,3-Dichlorobenzene, 1,4-Dichlorobenzene, 1,1-Dichloroethane, 1,2-Dichloroethane, 1,1-Dichloroethylene, *cis*-1,2-Dichloroethylene, *trans*-1,2-Dichloroethylene, Dichloromethane, 1,2-Dichloropropane, 1,3-Dichloropropane, 2,2-Dichloropropane, 1,1-Dichloro-1-propene, *cis*-1,3-Dichloropropene, *trans*-1,3-Dichloropropene, Ethylbenzene, Hexachloro-1,3-butadiene, Isopropylbenzene, p-Isopropyl toluene, Naphthalene, Propyl benzene, Styrene, 1,1,1,2-Tetrachloroethane, 1,1,2,2-Tetrachloroethane, Tetrachloroethylene, Toluene, 1,2,3-Trichlorobenzene, 1,2,4-

Trichlorobenzene, 1,1,1-Trichloroethane, 1,1,2-Trichloroethane, Trichloroethylene, 1,2,3-Trichloropropane, 1,2,4-Trimethylbenzene, 1,3,5-Trimethylbenzene, *o*-Xylene, *m*-Xylene, *p*-Xylene) supercritical CO_2, hydro matrix, heptanes and Nitrogen gas (N_2) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA)

Ultra pure water (18.2 MΩcm) for preparation of reagents was obtained from a MilliQ system supplied by Milford (Mass, USA).

Truffles were purchased from local markets (Kokotsha, Botswana).

3.3 Equipment and materials

Evaluation of the morphology of samples was done by employing a Vega Tescan scanning electron microscope (SEM).

Pressurized hot water extraction (PHWE) was performed in a pressurized static batch reactor with a cell volume of 100 mL supplied by Autoclave Engineers (Erie, USA). The reactor was comprised of; a heating unit, stirrer, inlet N_2 gas, and a filter for both sample inlet and outlet valves.

Dynamic hot water extraction was performed using an in-house built system made of an adopted old GC oven (Fig 4.4.4) comprising; HPLC pump, stainless steel tubing, sample cells (with inlet and outlet filters), pressure regulator and sample collection outlet.

Characterisation of samples by Fourier Transform Infrared (FTIR) employed the use of a Perkin Elmer spectrum 100 FT-IR spectrometer.

A Liquid Chromatograph Mass Spectrometry (LCMS) employing a time of flight detector was used for fatty acids and amino acid qualitative analysis of the pressurized hot water extracts using negative TOF MS scanning mode and Electron Spray Ionisation (ESI) for positive identification.

Supercritical CO_2 extraction was carried out using an SFE ISCO instrument at constant temperature (40 °C) and pressure (150 bar). 10 cm^3 stainless steel sample cells were used for extraction. The SFE ISCO instrument set was comprised of; controller, water bath, pump, oven, restrictor, CO_2 source, and stainless steel sample cell.

Four solid phase micro extraction fibers of different polarities were employed; PDMS 100 µm, PDMS 7 µm, Polyacrylate 85 µm and CAR/DVB/PDMS 50/30 µm.

The SPME materials (fibers and manual holder) were obtained from SUPELCO, (Bellefonte, USA).

3.3.1 Selection of best sample matrix for optimal separation of truffle volatile compounds by gas chromatography –head space-solid phase micro extraction (GC-HS-SPME)

Separation of volatile compounds

Chromatography was performed by a GC Varian 3800 using CP-Sil 8 CB capillary column 30 m x 0.25 µm x 0.32 mm for separation. The GC was coupled to a Varian COMBI PAL headspace sampling system. The same optimal conditions were adopted for screening samples using an Agilent GC 6890N with a ZB-5Msi 30 m x 0.25 mm x 0.25 µm column.

Best fiber selection for separation

A Varian GC 3800 was used employing a VF-5ms 30 m x 0.25 mm x 0.25 µm column to separate volatile organic compounds desorbed from the four solid phase micro extraction fibers (PDMS 100 µm, PDMS 7 µm, Polyacrylate 85 µm and CAR/DVB/PDMS 50/30 µm). Number of peaks, and degree of separation was observed for different fibers. The programme worked on splitless mode for a run time of 60 min. The oven programme was set at 40 °C ramping to 60 °C at 10 °C /min and holding for 2 min then ramping to 200 °C at 3 °C /min and holding for 9 min. The injector was set at 250 °C on a split less mode.

Identification of compounds

A Varian GC 3800 was used for separation of volatile organic compounds and a Varian 2000 MS detector using electron ionization mass spectrometry (EI-MS) fragmentation was employed for positive identification. Compounds identities were confirmed using the Saturn Library search.

3.4 Sample preparation

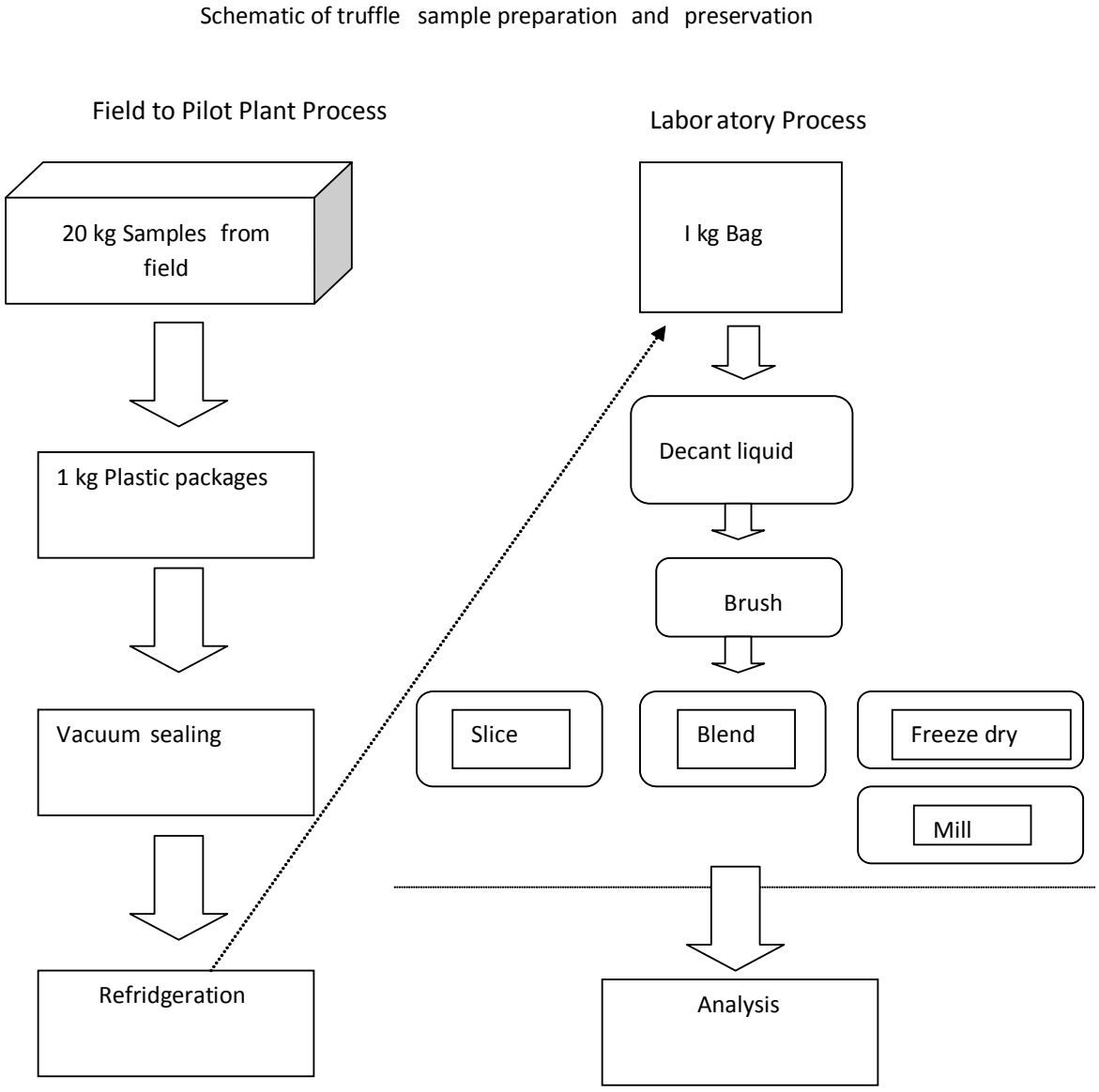


Fig. 3.4.1 Sampling of the Kgalagadi desert truffles

3.4.1 Sampling of truffles

Clean samples were packaged into transparent plastic bags in batches of 1 kg (Fig 3.4.1). The bags were then vacuum sealed and put into a cold room for storage. When required, a bag was retrieved for laboratory tests. Samples from the 1 kg bag were drained of excess liquid which had come up as a result of the pressure exerted on the package as air was sucked out during vacuum sealing. The liquid was stored in a refrigerator and kept for analysis. Excess soil was brushed off and clean truffle samples were subsequently subjected to different

preparative steps for analysis. 500 g of the 1kg batch was put into a blender and homogenised to a smooth paste. Some of the paste (about 100 g) was analysed fresh for volatile compounds using GC-HS-SPME and the remaining 450 g were frozen overnight and transferred to a freeze drier. The freeze dried sample was milled to a fine powder with a laboratory cyclone mill, the sample was then transferred into a plastic bag and vacuum sealed before storage in a refrigerator. The remaining 500 g fresh truffle sample was kept clean to provide small slices of truffles for volatile compounds analysis using GC-HS-SPME. From the procedure, four different forms of truffle samples were obtained namely; sliced, freeze dried and milled into powder, fresh truffles blended into paste, and decant liquid from vacuum sealed bags after refrigeration. These were kept for GC-HS-SPME work to evaluate the matrix effect on separation of volatile organic compounds from truffles.

3.4.2 Evaluation of truffle morphology by a scanning electron microscope (SEM)

This procedure was carried out in order to address the fact that truffle morphology and composition differs due to environmental factors. This is in line with objective 1 of addressing the physical characterization of the Kgalagadi desert truffle morphology by scanning electron microscopy. Fresh truffle slices were obtained by slicing the truffle into half with a scalpel. Thin slices were removed from the outer, the central (heart of the truffle) and the inner skin. The slices were then dipped into a solution of 2.5% glutaraldehyde in 0.1M phosphate buffer and allowed to soak overnight. On day two, the slices were each immersed for 10 min in a solution of 0.1 M sodium phosphate buffer. This procedure was repeated once for each sample. The samples were then taken through treatments of an alcohol assay in an ascending order of concentration at 5 min exposure in each i.e. 30%, 50%, 70%, 80%, 90% and absolute alcohol. Samples were then transferred for critical point drying for 2 h. After CO_2 critical point drying, slices were taken for gold spattering. Gold covered slices of the truffle were then taken for observation under a scanning electron microscope. The morphology of all the slices from different locations of the truffle was studied. To improve resolution samples were treated in a solution of hexamethyldisilazane for 1 h then re-observed under SEM.

3.4.3 Pressurized hot water extraction for truffles using a static batch reactor

All procedures stated in subsections 3.4.3 to 3.4.6 were executed in the light of green chemistry to optimize and promote extraction techniques which are safe to the environment whilst addressing the 2nd and 3rd objectives of the study; The analysis of extracts by FTIR to profile functional group components of truffle oil and PHWE extracts and the effectiveness of green technology extraction yield of truffle volatiles for a method based on supercritical fluid extraction.

A 9 g truffle sample was weighed into a 100 ml stainless steel cell. 90 cm^3 deionized water was added to the sample and taken for extraction in the mini reactor (Fig 3.4.4.1). The temperature for the reactor was set at 110 °C. The pressure inside the reactor was 1 – 3 bars.

An extraction time of 20 min was used. 6 min was allowed for equilibration prior to setting extraction time. The sample extract was collected into amber HPLC vials (2 mL) by turning the outlet valve, allowing the over pressure to push out the extract into the vial. The first 1 mL collected was discarded to clean the tubing. A round 4 mm micropore metal filter (10 µm) was built into the fitting for filtration of the extraction solvent (H_2O) before collection.

Clean extracts were collected into 1 ml amber HPLC vials and taken for analysis with LCMS.

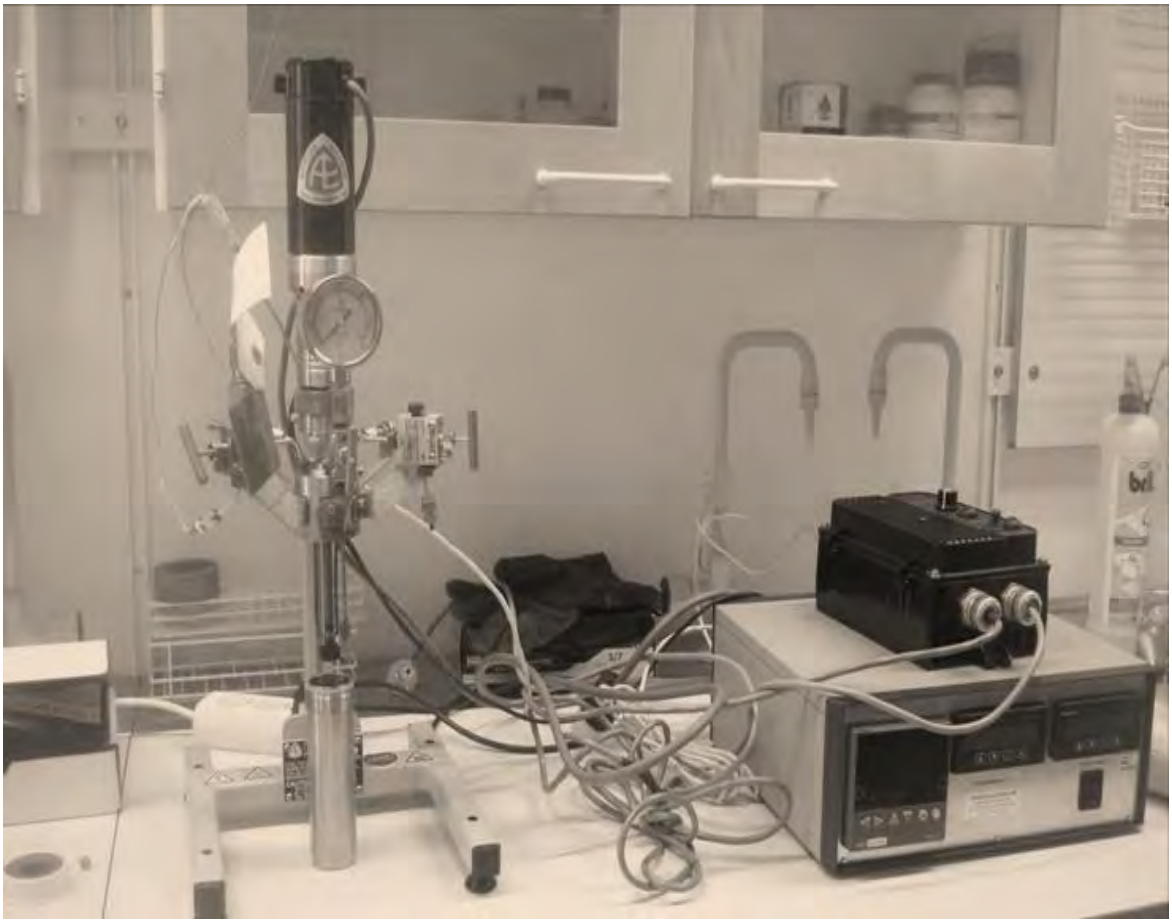


Fig. 3.4.4.1 A batch system used for PHWE

3.4.4 Pressurized hot water extraction for truffles using a dynamic system

3 g fresh truffle was mixed with 3 g sea sand and ground for homogeneity and to avoid clogging of the sample cell. The mixture was transferred into a stainless steel extraction cell and placed in the PHWE oven (Fig 3.4.5.1). Deionised water was the extraction solvent employed to fill the extraction cell and preheated for 5 min in the static mode to equilibrate. The extraction temperatures were 60 °C, 80 °C, 100 °C and 120 °C. Pressure was regulated through an adopted HPLC pump at a flow rate of 1 ml/ min. Extracts were collected in 10 mL glass vials and characterized by FTIR.

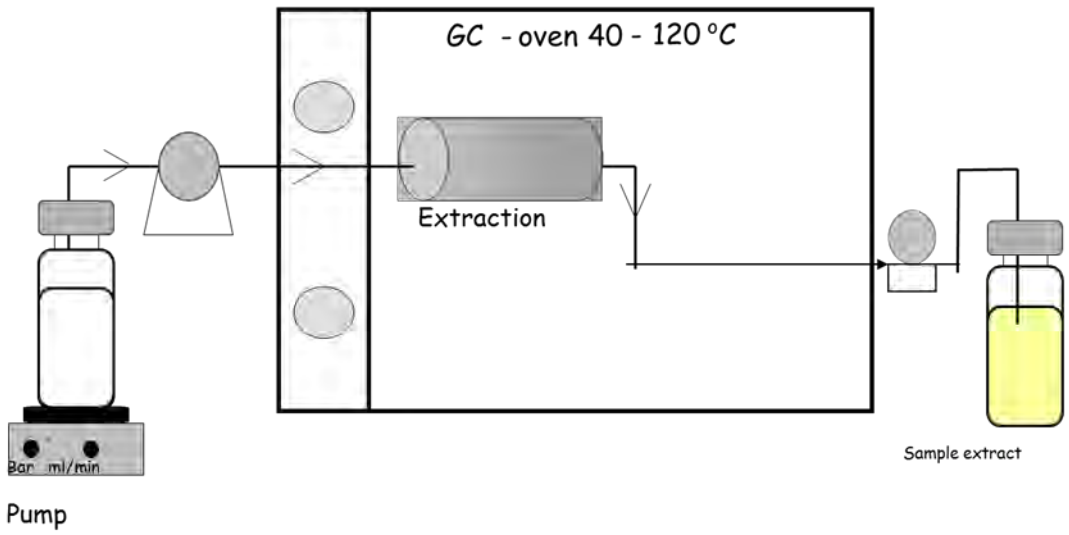


Fig. 3.4.5.1 Pressurized hot water extraction -dynamic system

3.4.5 Characterization of truffles by fourier transform infrared spectrometer (FTIR).

The problem of truffle oil adulteration calls for quick and robust monitoring tools, FTIR spectral analysis presents itself as a reliable non-destructive method to address this and hence objective 2 of the study; The analysis of extracts by FTIR to profile functional group components of truffle oil and PHWE extracts.

Fresh truffle slices were obtained by slicing the truffle into half with a scalpel. Thin slices were obtained from different sections of the truffle especially the central and the inner skin. Truffle slices were measured in their fresh form. Paste samples were obtained by grinding thin slices into a paste using pestle and mortar.

Half of the slices obtained of fresh truffles were dried in an oven at 70 °C overnight. Pestle and mortar were used to crash these into powder and a small portion used for FTIR measurement.

The decant truffle liquid was also taken in a small vial for measurement.

A soxhlet extract (previously extracted oil using petroleum ether) of the truffle was also taken for the FTIR analysis.

Different truffle samples were passed through the dynamic PHWE system (Fig 3.4.5.1) at temperatures of 60 °C and 80 °C, 100 °C, 120 °C and extracts collected.

An FTIR spectrum of each type of sample described above, including distilled water used for rinsing the sample compartment, was obtained by scanning a tiny amount of each sample on the sample compartment of Perkin Elmer 100 FT-IR spectrometer. The procedure was also performed for truffle defatted by soxhlet extraction and powdered freeze dried truffles.

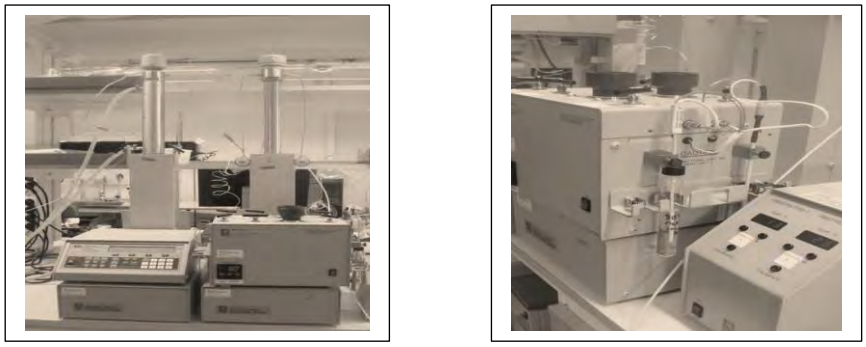
3.4.6 Super critical fluid extraction for truffles using SFE-ISCO instrument

The effectiveness of green technology extraction yield of truffle volatiles for a method based on supercritical fluid extraction was evaluated. The first part of SFE work aimed at evaluating the percentage yield of volatile organic compounds from truffles. A 1 g slice of truffle sample was cut using a scalpel. The sample was put in a pestle and mortar and then mixed with 2 g hydromatrix (1: 2 truffle/hydro matrix) in 10 cm^3 stainless steel sample cells and ground into a fine flowing powder for homogeneity. The mixture was then transferred into a stainless steel 10 ml sample cell. Extraction times were 5, 10, 15 and 20 min in the dynamic mode. Supercritical CO_2 extraction yield was measured at constant temperature (40 °C) and pressure (150 bar).

Temperature and pressure of extraction were varied to evaluate the effect on extraction. The restrictor flaw rate was set at 1.5 ml/min collecting into 4 cm^3 heptane.

The second part of SFE work focused on extraction and analysis of volatile compounds by GC for separation. A 1 g slice of truffle sample was cut using a scalpel. The sample was put in a pestle and mortar and then mixed with 2 g hydromatrix (1: 2 truffle/hydro matrix) in 10 cm^3 stainless steel sample cells and ground into a fine flowing powder for homogeneity. The mixture was then transferred into a stainless steel 10 ml sample cell. Extraction time was 20 min in the dynamic mode at constant temperature (120 °C) and pressure (200 bar). The restrictor flaw rate was set at 1.5 ml/min. This experiment was performed for two different collecting solvents i.e. dichloromethane and isopropanol at 4 cm^3 each. The extracts were kept for separation with GC.

Further work on SFE truffle volatile extracts collected into dichloromethane and isopropanol involved the use of SPME PDMS 100 µm fiber prior to and after SFE on the same amount of truffle sample. Samples were taken for volatile organic compounds separation using GC. Standard dilutions were made from the EPA 524 VOC Mix A. 20 µg / ml and 200 µg / ml in methanol were injected into the GC using 1 ml syringe and monitored for volatile separation. The following conditions were used; column oven programme temp 80 °C, hold 5 min, 230 °C at 20 °C / min, hold 20 min, total run 32.50 min. The column used was ZB-5Msi, 30 m x 0.25 mm x 0.25 µm capillary column. These experiments were conducted at Lund University (Lund, Sweden), using the set up illustrated on Fig 3.4.3.1 (a) and (b).



(a) (b)

Fig. 3.4.3.1 SFE-ISCO Instrument (a) pressure pump with controller, (b) oven and restrictor

3.4.7 Qualitative analysis of amino acids and fatty acids of truffles using time of flight liquid chromatography mass spectrometry (TOF-LCMS)

PHWE has been applied in agricultural products in extracting bioactive compounds [64]. In order to ascertain the essential profiles of these compounds the samples should be analysed using fresh extracts to obtain full spectrums and reliably identify the compounds. The LC-MS was the method of choice to for objective 5 of this work to address the profile of bioactive compounds of the truffle oil and identify the composition of PHWE extracts by LCMS or GCMS.

The hot water extract sample was collected after 20 min from the mini reactor and filtered through a 0.45 µm nylon filter before screening by LC-MS. A 5 µL syringe was used for injection into the TOF LCMS API QSTAR PULSAR for amino acid and fatty acid qualitative analysis. Three scans were run for different molecular weight ranges of the amino acids and fatty acids respectively. Corresponding masses associated with amino acids and fatty acids were identified from the scans. The mass range scanned for fatty acids was 110-400 amu and for amino acids 70-187 amu respectively. Electron Spray Ionisation (ESI) was used for positive identification of compounds.

3.4.8 Selection of best sample matrix for optimal separation of truffle volatile compounds by gas chromatography –head space-solid phase micro extraction (GC-HS-SPME)

In line with the fourth objective of the study, extraction of volatile compounds with headspace solid phase micro extraction (HS-SPME) fibers was conducted.

This first stage of the extraction was applied to all four different truffle samples; liquid, freeze dried, paste and sliced truffle. Volatile compounds separation was first carried out using a Varian GC 3800 coupled to a COMBI PAL automatic sampling system with a 1 ml headspace syringe set at a temperature of 50 °C. A 0.5 g of each sample was weighed into a vial, crimped and taken for separation with GC. The exercise was carried out as a method development procedure to evaluate the optimum sample matrix conditions for a good signal due to volatiles. The detection mode was flame ionisation. CP-Sil capillary column 30 m x 0.25 µm x 0.32 mm was used under the following conditions; column oven programme temp 80 °C, hold 5 min, 230 °C at 20 °C / min, hold 20 min, total run 32.50 min using an FID detector.

The second stage of the extraction involved the use of an SPME fiber PDMS 100 µm for different truffle matrix. The fiber was conditioned prior to use according to the manufacturer's instructions (see appendix 1). A 10 g truffle sample was weighed into a specially designed (glass blown) round bottom flask (Fig 3.4.9.1) for the extraction and concentration of volatiles. The sample was placed in a water bath at 50 °C and allowed to equilibrate for 5 min. An SPME fiber was then exposed through a rubber stopper on the neck of the flask and allowed to adsorb for 10 min before retraction and desorption into a GC injection port. The fiber was then exposed and allowed to desorb for 3 min. The run time for separation of compounds was conducted for 60 min. The detection mode was flame ionisation using Agilent GC 6890N with ZB-5Msi, 30 m x 0.25 mm x 0.25 µm capillary column. The following conditions were used; column oven programme temp 80 °C, hold 5 min, 230 °C at 20 °C / min, hold 20 min, total run 32.50 min.

3.4.9 Best fiber selection for separation - sample preparation of truffles for qualitative analysis of volatile organic compounds using GC-MS-HS-SPME procedure

A 10 g truffle sample was weighed into a specially designed (glass blown) round bottom flask (Fig 3.4.9.1) for the extraction and concentration of volatiles. The sample was placed in a water bath at 50 °C and allowed to equilibrate for 5 min. An SPME fiber (pre conditioned as per appendix 1) was then exposed through a rubber stopper on the neck of the flask and allowed to adsorb for 10 min before retraction and desorption into a GC. The fiber was then exposed and allowed to desorb on the GC injector port for 3 min. Analysis was conducted for 60 min. Blank signals of the pre-conditioned fibers were obtained prior to extraction. The following conditions were used; column oven programme temp 80 °C, hold 5 min, 230 °C at 20 °C / min, hold 20 min, total run 32.50 min. ZB-5Msi, 30 m x 0.25 mm x 0.25 µm capillary column was used with FID as the detection mode.

Chromatograms of the volatile compounds were obtained using a Varian GC 3800 for extracts from four different fibres (PDMS 100 µm, PDMS 7 µm, Polyacrylate 85 µm and CAR/DVB/PDMS 50/30 µm). The outputs were evaluated for best separation.

Fig. 3.4.9.1 Sampling for volatile organic compounds using SPME

3.4.10 Identification of compounds

A 10 g truffle sample was weighed into a specially designed (glass blown) round bottom flask (Fig 3.4.9.1) for the extraction and concentration of volatiles. The sample was placed in a water bath at 50 °C and allowed to equilibrate for 5 min. A PDMS 100 µm SPME fiber (pre conditioned as per appendix 1) was then exposed through a rubber stopper on the neck of the flask and allowed to adsorb for 10 min before retraction and desorption into a GC-FID. The fiber was then exposed and allowed to desorb on the GC injector port for 3 min. Analysis was conducted for 60 min. Blank signals of the pre-conditioned fiber were obtained prior to extraction. The following conditions were used; column oven programme temp 80 °C, hold 5 min, 230 °C at 20 °C / min, hold 20 min, total run 32.50 min. The column used was ZB-5Msi, 30 m x 0.25 mm x 0.25 µm capillary column.

Chromatograms of the volatile compounds were separated by a Varian GC 3800 coupled to a Varian 2000 mass detector. Electron ionization mass spectrometry (EI-MS) fragmentation was employed for positive identification. Compounds identities were confirmed using the Saturn Library search. The data was used as a profile of the volatile compounds in the Kgalagadi desert truffle.

Chapter 4

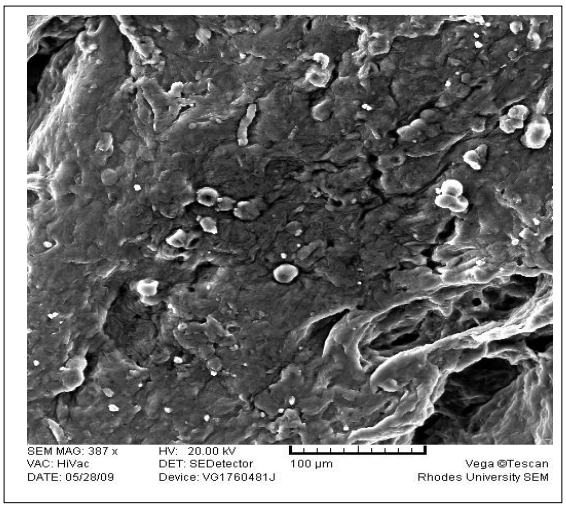
4 Results and discussions

4.1 Overview

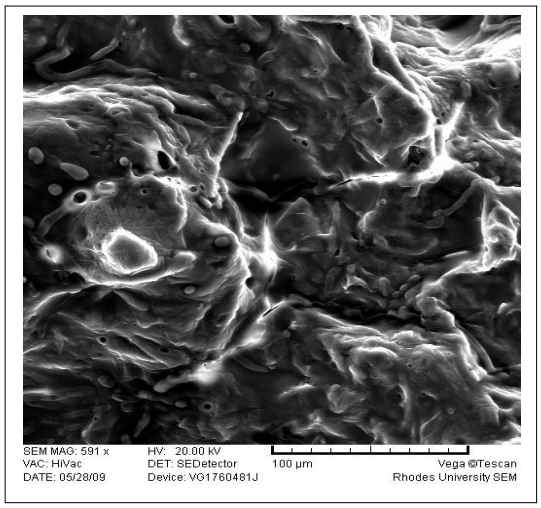
This section of the thesis is intended to bring into perspective, results of the study and discussions emanating from all analytical work carried out as outlined in the experimental section. An effort is made to align the methods and compare findings with the current trends and systems used in literature. In the light of green chemistry, the methods used for this study were all carefully selected to qualify them as safe both to the user and the environment. The section starts by discussing the physical characterization parameters of the Kgalagadi desert truffle in an effort to establish its uniqueness from other varieties of truffles around the world. Morphology studies were performed to this effect employing a scanning electron microscope whilst the oil and pressurized hot water extracts were characterized using fourier transform spectroscopy. The green chemistry technologies of SFE and PHWE and their application in the extraction of volatiles and bio-active compounds (fatty acids and amino acids) are also evaluated. The use of LCMS to profile PHWE extracts for bioactive compounds is discussed. The main component of this section discusses the results for the qualitative analysis of volatile compounds in truffle using another green, solventless technique of SPME. The concluding discussion focuses on volatile compound separation by GC and identification using GCMS and the associated library search.

4.2 Evaluation of truffle morphology by a scanning electron microscope (SEM)

Morphological studies are essential in species identification, as the position of spores is a tool used to determine dispersion hence multiplication of plant species [65]. The observation of spores is necessary for species identification, although they are not characteristic only to mushrooms. A study of spores is usually done with an optical microscope. However, optical microscopic observation is limited in the case of colourless or thick tissues. A scanning electron microscope (SEM) may compensate for the limitations associated with an optical microscope because it is possible to observe colourless tissues well and it provides three-dimensional images. Truffle heart sample showed pockets of spores the size of 2, 5 μm (see Fig 4.1.1 C-E). There was no sign of spores on the outer and inner skin samples. The colour of the spores could not be determined.



(A)



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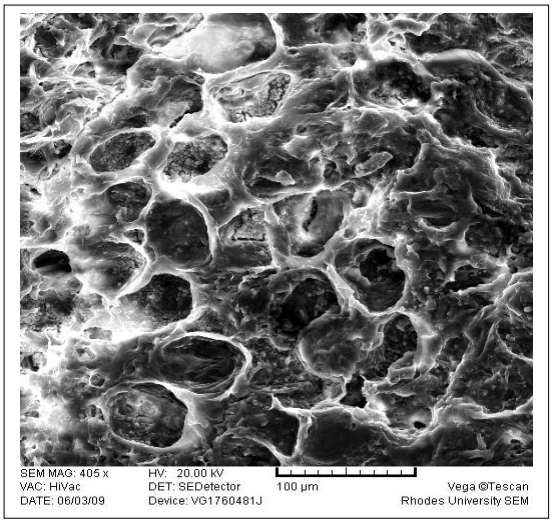
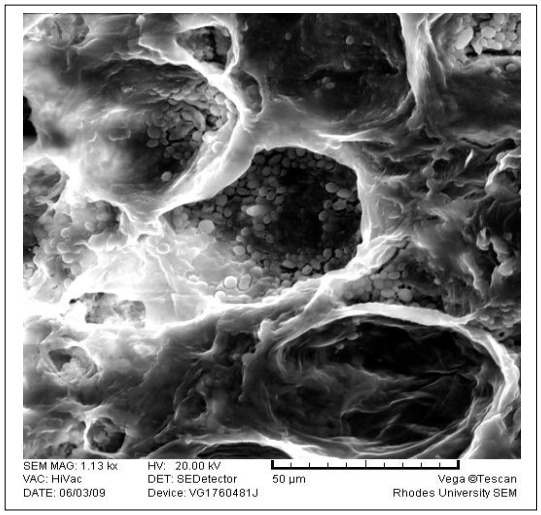
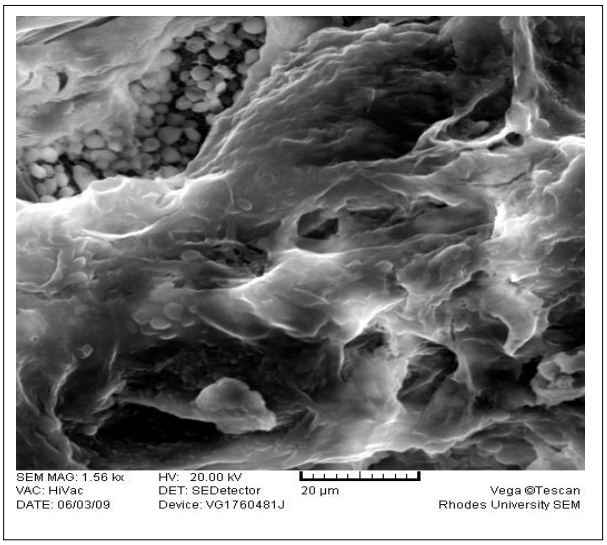


(C)



(D)



(E)

Fig. 4.1.1 Truffle morphology by SEM; (A) - outer skin, (B) -inner skin, (C), (D) & (E) -heart

The result suggests that as per literature [4] truffles multiply through animal dispersion as spores were found deep in the middle of the fruiting body and nothing immediately under the skin and the outer part. The study initially proved a challenge due to poor resolution. This was attributed to the gummy nature associated with esters in the sample. Hexamethyldisilazane was then used to treat the samples for an hour after which they were re-observed under the SEM with improved resolution. The morphology of truffles is an important quality attribute that could be employed for species identification and help curb adulteration for business gains.

4.2.1 Pressurized hot water extraction for truffles using a static batch reactor

Clean extracts were collected into 1 ml amber HPLC vials and taken for analysis with LCMS.

4.2.2 Pressurized hot water extraction for truffles using a dynamic system

Pressure was successfully regulated through an adopted HPLC pump at a flow rate of 1 ml/ min. Extracts were collected in 10 mL glass vials at temperatures of 60 °C, 80 °C, 100 °C and 120 °C and characterized by FTIR.

4.2.3 Characterization of truffles by fourier transform infrared spectrometer (FTIR).

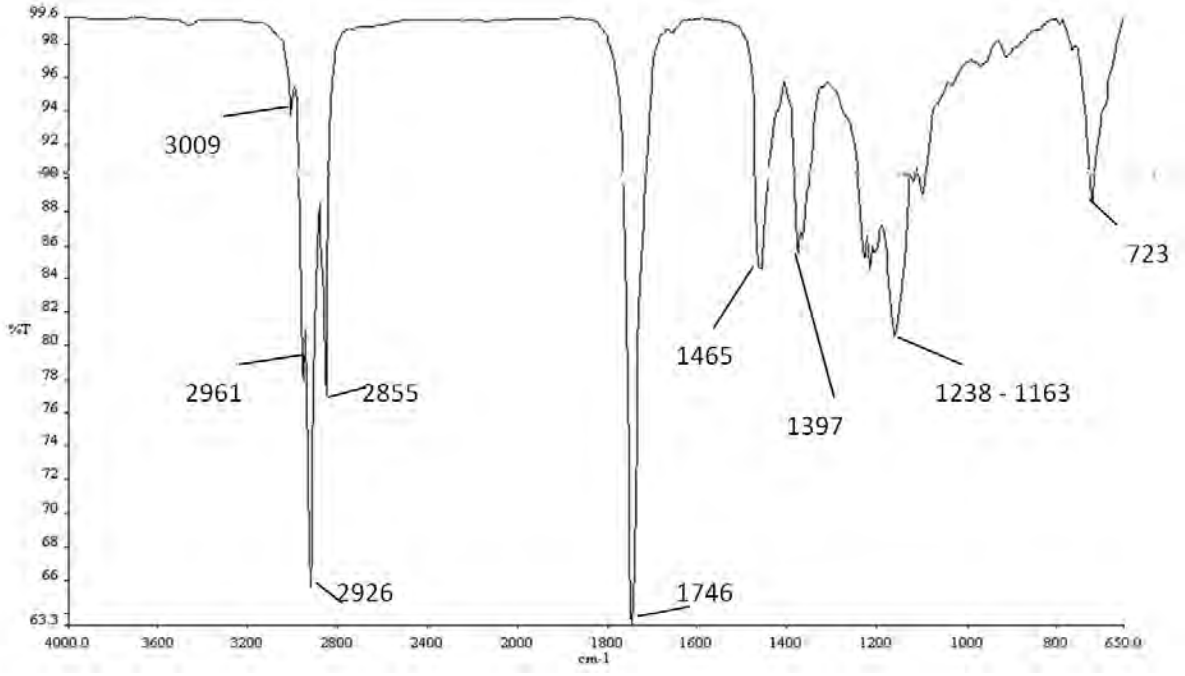


Fig. 4.2.1 (a) IR spectra of truffle oil after soxhlet extraction

The oil extract spectrum could be used as a fingerprint to confirm origin and integrity of truffles. Due to the expensive nature of real truffle oil, it is commonly obtained as a synthetic product. FTIR provides a quick way of assessing and evaluating the quality of real truffle oil associated with particular species.

This raises the need to know the FTIR spectra of real truffle oil (Fig 4.2.1a) and its fatty acids composition as observed under table 4.5.2. The functional groups of interest in truffle analysis are those that can give an indication of the freshness or signs of adulteration. A band shift observed at 3009 cm^{-1}, which is typical of C – H stretching vibration of the cis – double bond allows for the determination of oil adulteration [66]. Fresh truffle oil would normally display strong absorption bands at 1077 -1040 cm^{-1} that are associated with C-O stretching in carbohydrates. These vibration structures depict polysaccharide and protein content of the truffles. The study results confirmed that fat extraction of the truffle does not affect protein and carbohydrate content as shown by peak 1077 cm^{-1}-1040 cm^{-1} of the defatted sample. Other studies have shown that moldy and healthy truffles could be distinguished by protein bands [70].

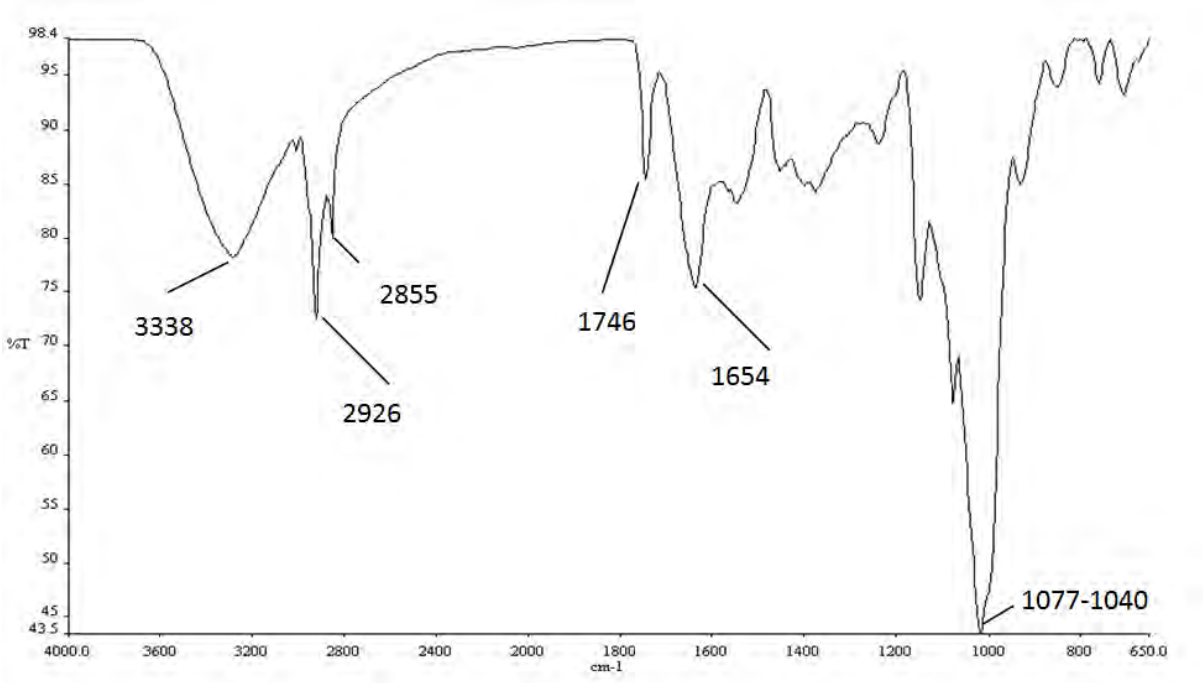


Fig.4.2.1 (b) IR spectrum of defatted truffle

The defatted truffle sample was ran for comparison to evaluate the effectiveness of soxhlet extraction.

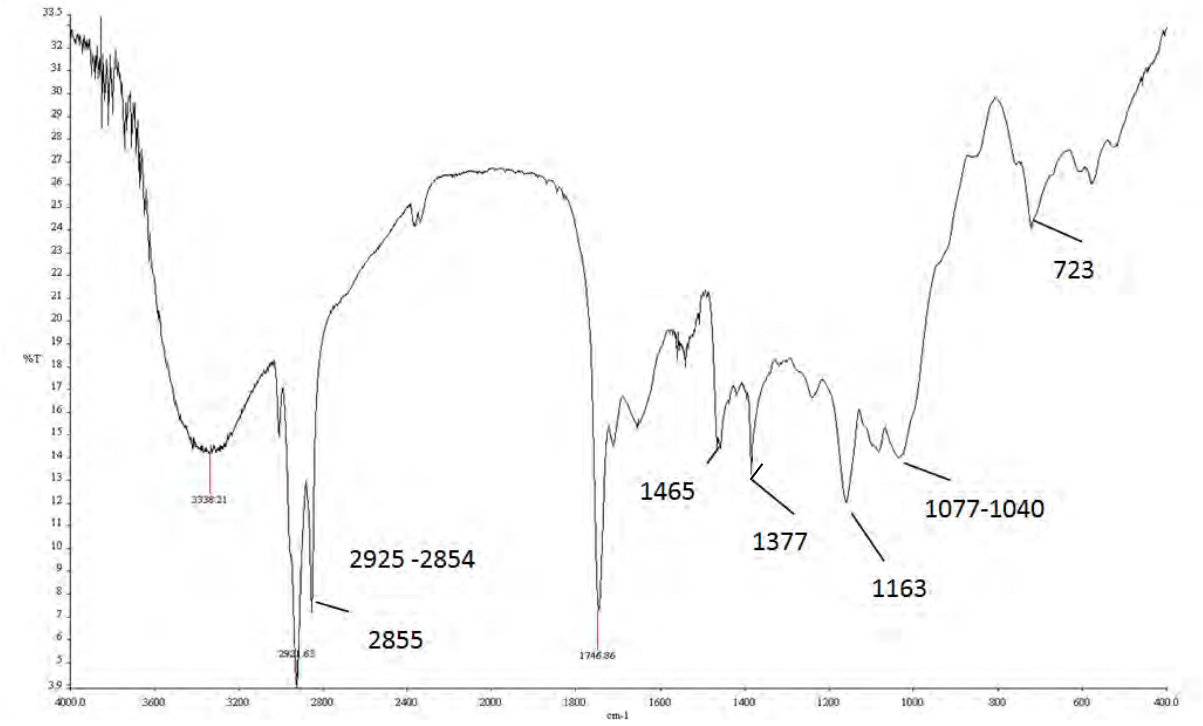


Fig. 4.2.1 (c) IR spectra of truffles dried at 70 °C overnight

The sample dried overnight (Fig 4.2.1 c) showed prominent peaks of COOH at 3338 cm^{-1} and C=O at 1746.86 cm^{-1} respectively. The FTIR spectra of the desert truffle oil also had the main peak associated with the carbonyl group at 1745 cm^{-1} but there was no carboxylic acid functional group. There were also some unidentified groups at 1217 cm^{-1} and 1161 cm^{-1} which could be said to be characteristic of most edible oils [66]. Figures 4.2.1(c) and 4.2.1 (d) show the effect of processing on the truffle oil, the former affecting moisture removal and the later showing the effect of the presence of water introduced by the extraction process.

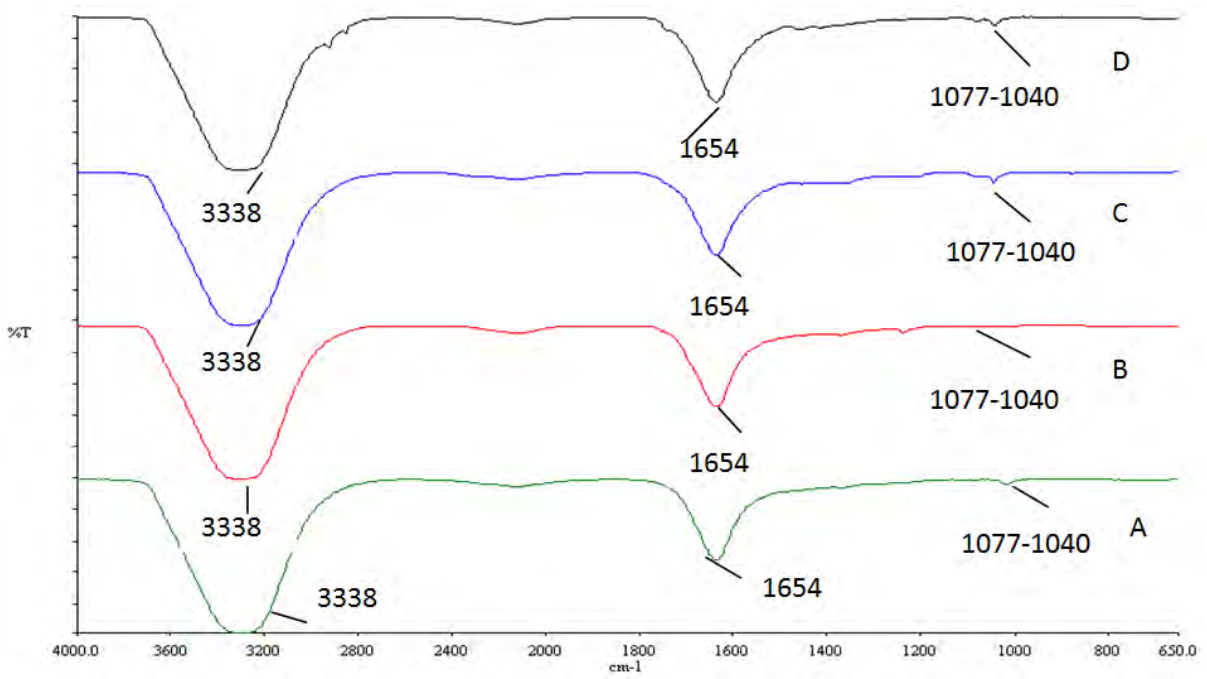


Fig. 4.2.1 (d) Liquid extracts from PHWE; 60 °C (A), 80 °C (B), 100 °C (C), and 120 °C (D)

There were a lot of structural similarities in the spectra observed in all the pressurized hot water extracts (Fig 4.2.1 d). The same pattern was observed for the distilled water. This suggested that the equipment could detect water from the fresh samples probably due to the truffle high moisture content > 70% [2]. The spectra consistently showed C-O stretching at 1077 cm^{-1} – 1040 cm^{-1} associated with carbohydrate content and a peak in the double bond stretching region typical of C=C stretching vibrations at 1654 cm^{-1}. All treatments showed the presence of the COOH functional group at 3338 cm^{-1}.

Processing by hot water and soxhlet extraction both proved not destructive to protein as shown by the prominent overlapping CH_2 vibrational peak on the defatted sample at 723

cm^{-1}.The application of FTIR in food research proves to be a robust, rapid and non-destructive method of choice to be applied prior to extraction of truffles. The different sample extraction and preparation protocols provided an opportunity to confirm that there is indeed no matrix effect when using FTIR.

Table 4.2.1 Evaluation of FTIR spectra of truffle extracts

Sample	**Region of Functional groups cm^{-1}**	***Interpretation of IR Spectrum***
Truffle oil, soxhlet extract	1. 3009	Hydrogen stretching region, C-H stretching vibration of the cis-double bond (=CH)
	2. 2961 and 2855	Hydrogen stretching region, Symmetric and asymmetric stretching vibration shoulder of the aliphatic CH_3 group
	3. 2926	Hydrogen stretching region, symmetric and asymmetric stretching vibration of the aliphatic CH_2 group.
	4. 1746	Region of double bond stretching, Ester carbonyl functional group (C=O) of the triglycerides
	5. 1465	Region of bond deformation and bendings, bending vibrations of CH_2 and CH_3 aliphatic groups
	6. 1397	Region of bond deformation and bendings, bending vibrations of CH cis- olefinic groups
	7. 1238 – 1163	Fingerprint region, Stretching vibrations of C – O ester groups
	8. 723	Fingerprint region, overlapping of CH_2 vibration and cis-disubstituted olefins
Defatted truffle	1. 3338	COOH
	2. 2926	Hydrogen stretching region Symmetric and asymmetric

		stretching vibration of the aliphatic CH_2 group.
	3. 2855	Hydrogen stretching region Symmetric and asymmetric stretching vibration shoulder of the aliphatic CH_3 group
	4. 1746	Region of double bond stretching, Ester carbonyl functional group (C=O) of the triglycerides
	5. 1654	Region of double bond stretching, C=C stretching vibrations
	6. 1077 - 1040	C-O stretching in carbohydrates
Truffle dried at 70 °C overnight	1. 3338	COOH
	2. 2925 – 2854	Hydrogen stretching region, Symmetric and asymmetric stretching vibration shoulder of the aliphatic CH_3 group
	3. 2985	Hydrogen stretching region Symmetric and asymmetric stretching vibration shoulder of the aliphatic CH_3 group
	4. 1746	Region of double bond stretching, Ester carbonyl functional group (C=O) of the triglycerides
	5. 1465	Region of bond deformation and bendings, bending vibrations of CH_2 and CH_3 aliphatic groups
	6. 1377	Region of bond deformation and bendings, bending vibrations of CH_2 groups
	7. 1163	Finger print region, C-O ester stretching
	8. 1077 – 1040	C-O stretching in carbohydrates
	9. 723	Fingerprint region, overlapping of CH_2 vibration and cis-disubstituted olefins
PHWE extracts 60 °C, 80 °C, 100 °C, and 120 °C	1. 3338	COOH
		Region of double bond stretching,

	2. 1654 3. 1077 - 1040	C=C stretching vibrations C-O stretching in carbohydrates

4.2.4 Super critical fluid extraction for truffles using SFE-ISCO instrument

Table 4.4.1 Evaluation of truffle volatiles yield after SFE extraction at various conditions of temperature and pressure

Temperature (°C)	Time (min)	% Yield
60	5	1.7
60	10	5.1
60	15	0.1
60	20	1.5
80	5	0.1
80	10	0.2
80	15	1.0
80	20	0.2
120	5	1.2
120	10	2.0
120	15	3.1
120	20	13.1
140	5	1.3
140	10	15.2
140	15	20.8
140	20	51.5

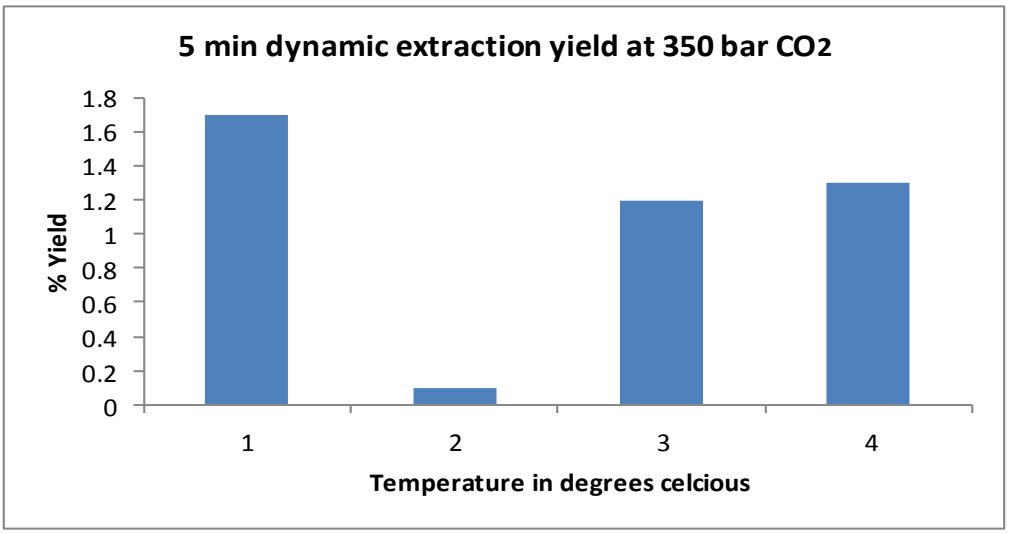


Fig. 4.4.1 Evaluation of truffle volatiles SFE :tion yield at various conditions of temperature and pressure

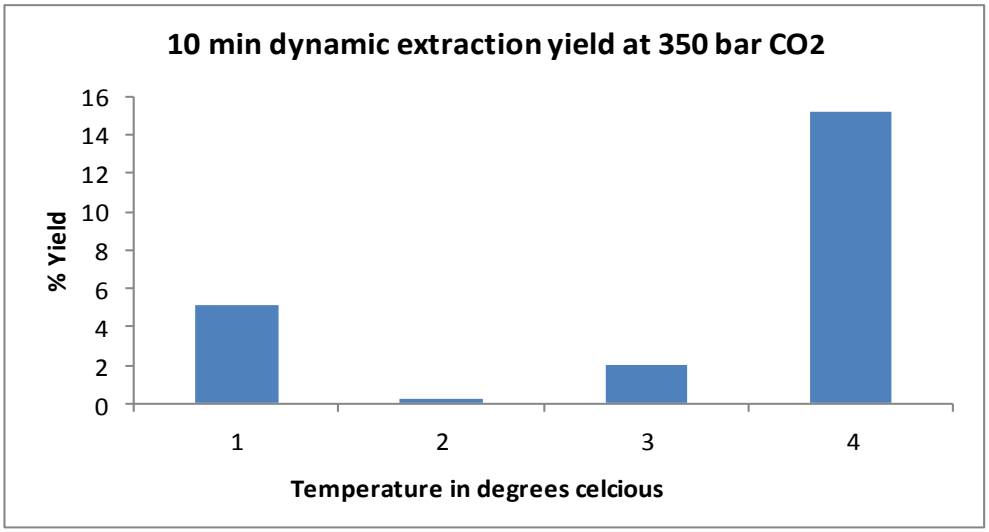


Fig. 4.4.2 Evaluation of truffle volatiles SFE extraction yield at various conditions of temperature and pressure

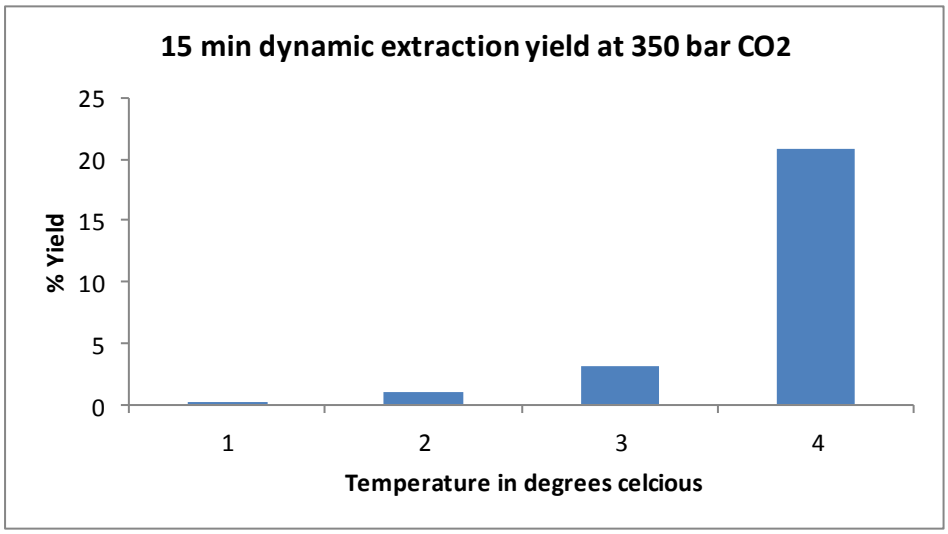


Fig. 4.4.3 Evaluation of truffle volatiles SFE extraction yield at various conditions of temperature and pressure

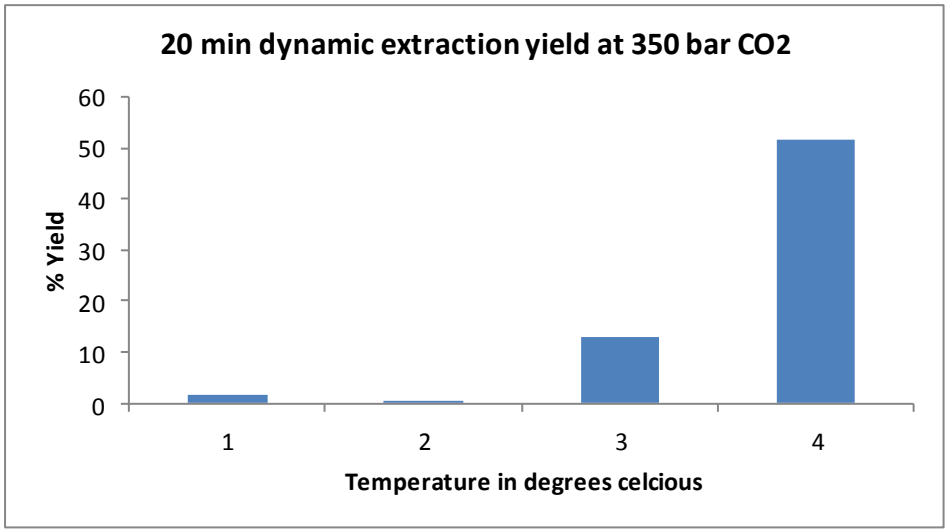


Fig. 4.4.4 Evaluation of truffle volatiles SFE extraction yield at various conditions of temperature and pressure

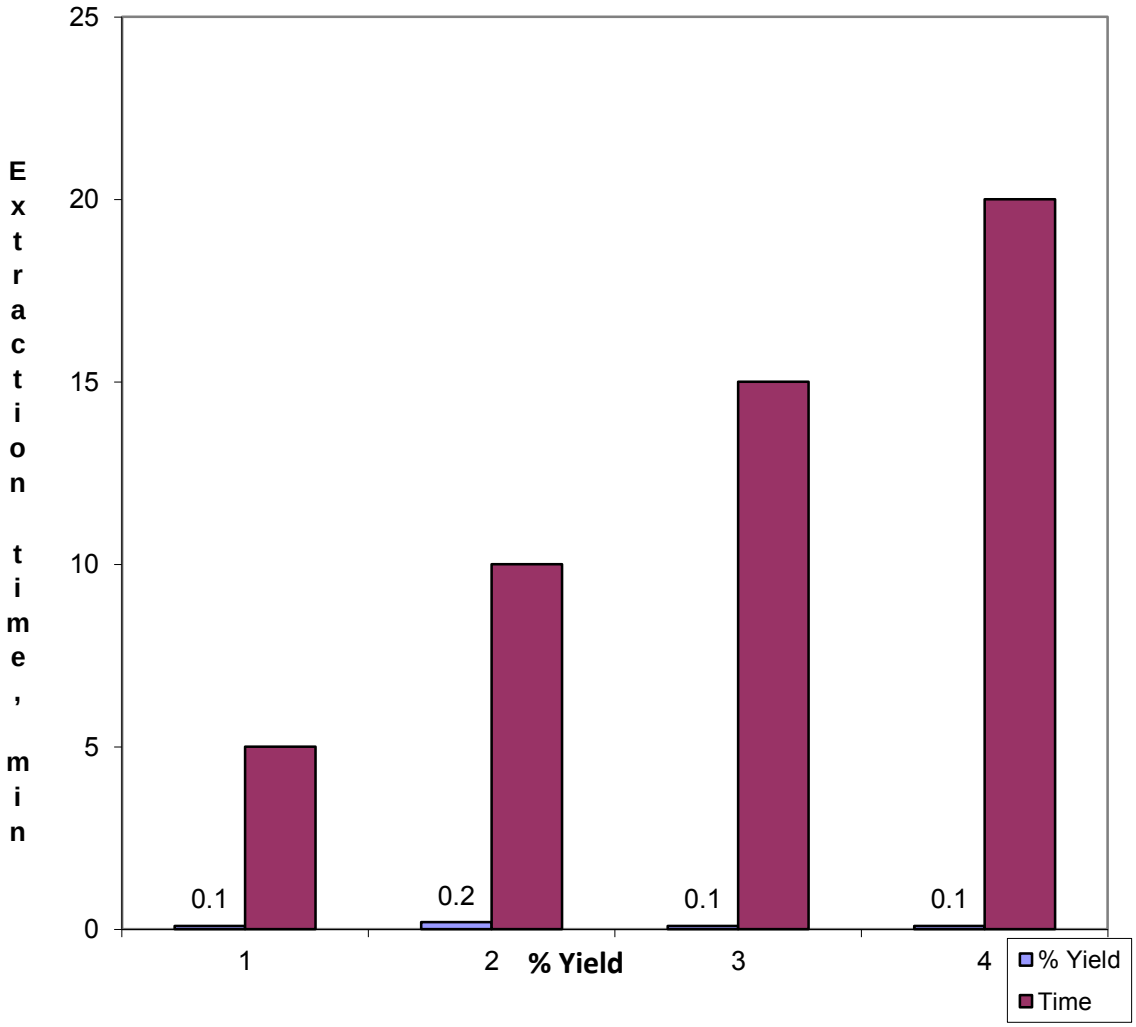


Fig. 4.4.5 Optimization of truffle volatile extraction yield

The percentage yield was very low with the highest value being 0.2% after an extraction time of 10 minutes (Fig 4.4.5) during optimization at 40 °C and 140 bar. Due to this fact, several temperature conditions were studied (Fig 4.4.1-4.4.4) where; 1 – 60 ºC, 2 – 80 ºC, 3 – 120 ºC, 4 – 140 ºC. It was observed that at higher temperatures of 120 °C and 140 °C, the collection vessel collected inconsistent amounts of gummy extracts which may not be volatile compounds. These could be attributed to total carbohydrates. 60 °C after 10 mins gave a better yield of 5.1 %.

The second part of SFE work focused on extraction and subsequent analysis of volatile compounds by GC for separation. SFE has been applied to volatile compounds before in agricultural and food processing wastes and by-products which in most cases include lipids and lipophilic components [64].

An effort was made to analyse SFE extracts for volatile compounds by GC without much success. Data is not shown because of poor spectra and no compounds could be further identified from the library of the GC-MS (see Appendix 2 for spectra)**.** SFE proved to be a challenge in the extraction of volatile compounds from truffles despite trials with variable flow rates and different collecting solvents. The GC conditions which had been optimized for the separation of volatile organic compounds could not attain any separation from SFE extracts.

However, when coupled with SPME, the results were better, that is; applying SPME before and after SFE. SPME could confirm that there was indeed some degree of extraction of volatile compounds by SFE. When SFE extracts were injected directly into the GC in different solvents; dichloromethane and iso-propanol no separation was seen from the collected truffle extracts. The two collection solvents (dichloromethane and iso-propanol) after extraction time of 20 min did not give a very good response for the truffle volatiles by GC, there was no separation of compounds.

This proved that matrix effect failed SFE as the method of choice for the analysis of volatile compounds in truffles and SPME emerged as the preferred extraction method. This was due to the fact that the percentage yield of the extracts was small (see Table 4.4.1). There was a suspected possible volatile compounds loss at the collection point or suboptimal analyte partitioning parameters occurred during extraction. Lack of derivatization of free fatty acids to the more volatile methyl esters affected detection sensitivity during separation by GC. Longer extraction time may have increased the yield.

4.2.5 Qualitative analysis of amino acids and fatty acids of truffles using time of flight liquid chromatography mass spectrometry (TOF-LCMS)

The results on evaluation of truffle oil and hot water extracts further informed the need for fatty acid and amino acid analysis. FTIR spectra for PHWE extracts and truffle oil (see Fig 4.2.1 (a) and 4.2.1 (d)) showed the presence of protein functional groups hence the need to profile the amino acids and fatty acids in the water extracts. The hot water extract sample was obtained from the mini reactor at 110 °C for 20 min using static extraction. An LCMS was employed to screen for these bioactive compounds. The API QSTAR™ Pulsar Hybrid LC/MS/MS System used is a high performance hybrid quadrupole time-of-flight mass spectrometer designed for protein identification and characterization. The system generates high quality MS and MS/ MS data from an electrospray ionization (ESI). Amino acid analysis was included under the pretext that the bulk of biological samples are usually due to protein. Previous studies of the Kgalagadi desert truffles had indicated that they contain 22 % crude protein (NFTRC unpublished data).

The qualitative identification of the bio-active compounds was carried out in full scan mode for both classes of analytes, (70 – 185 amu, 135-156 amu and 156- 187 amu) for amino acids screening (see Figs 4.5.1 (a), (b) & (c)). A total of 16 amino acids (Table 4.5.1) were identified. 63% of the amino acid composition were non-essential and the rest were essential amino acid namely; histidine, leucine, lysine, methionine, phenylalanine, threonine and valine. Glycine showed the highest peak intensity. Glycine is considered a glucogenic amino acid which supply the body with glucose needed for energy. It also helps regulate blood sugar levels, and thus glycine supplementation may be useful for treating symptoms characterized by low energy and fatigue, such as hypoglycemia, anaemia, and Chronic Fatigue Syndrome. The class of fatty acids was determined to give an indication of the composition of the highly priced truffle oil. The full scan mode was used in this order; 110 – 257 amu, 200 -300 amu and 300 – 400 amu for fatty acid screening (see Figs 4.5.2 (a), (b) and (c)). A total of 17 fatty acids (MW 132.12-367.49) were identified. The majority of the fatty acid composition was saturated structures. Four unsaturated fatty acids were identified as; palmitoleic, oleic, linoleic and α- Linolenic acid. Lignosteric acid (MW 367.49) was the most prominent with a peak intensity of 398.63. This saturated fatty acid is normally found in wood tar, various cerebrosides, and in small amounts in most natural fats.

Research shows that, plants, as do fungi, utilise their fatty acids to produce volatile compounds [71]. The main component of mushroom flavour is known to be 1-octen-3-ol. Its biosynthesis involves oxidation of lenoiec acid, which is also dominant in truffles [53,71].

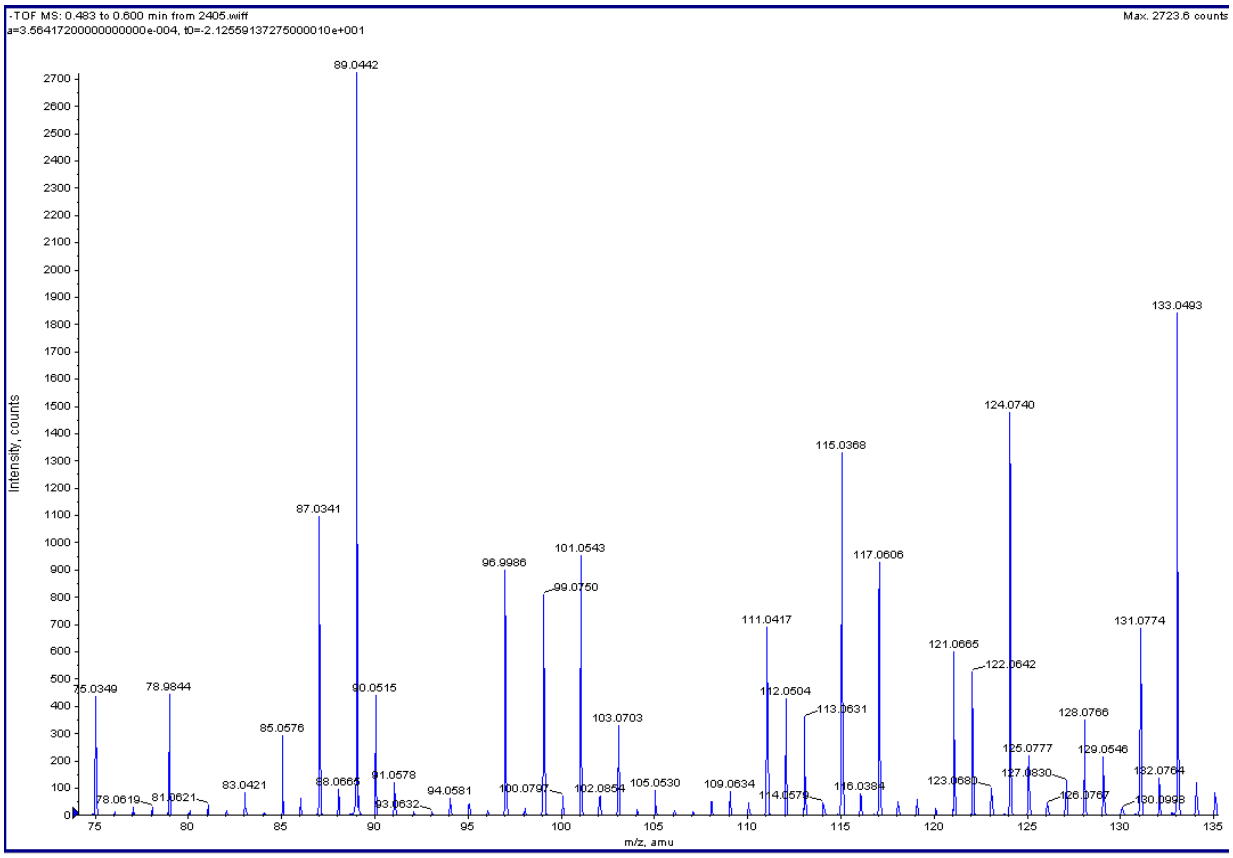


Fig. 4.5.1 (a) Full scan LCMS profiling of amino acids in truffles

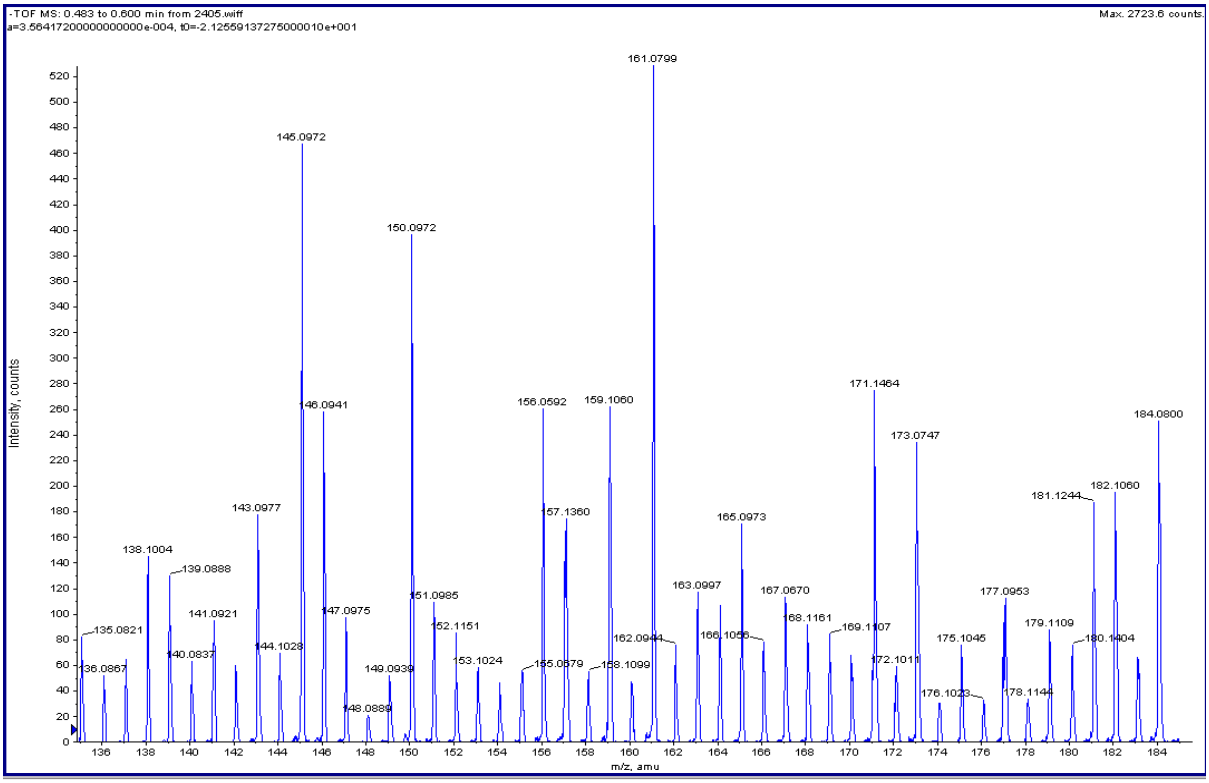


Fig. 4.5.1 (b) Full scan LCMS profiling of amino acids in truffles

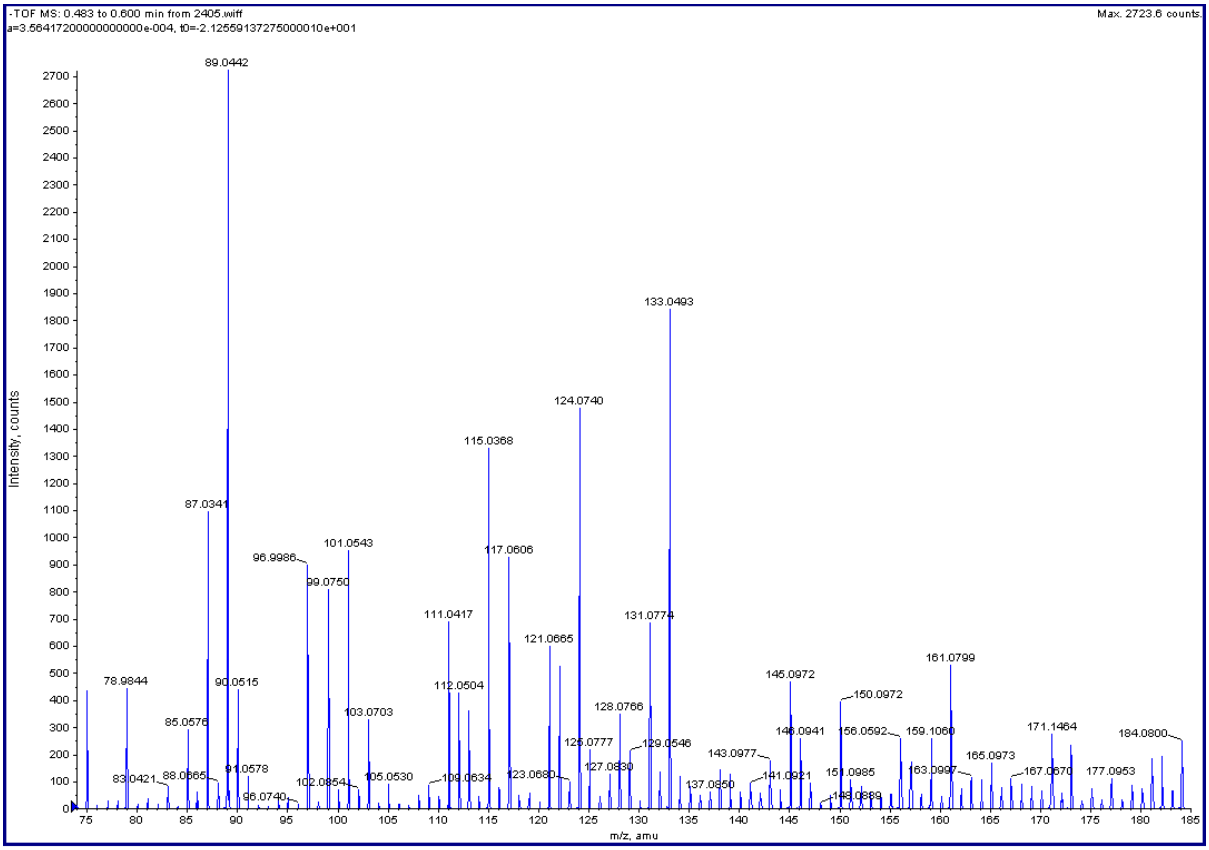


Fig. 4.5.1 (c) Full scan LCMS profiling of amino acids in truffles

The electron spray ionisation (ESI) mode was used. ESI-MS like all other mass spectrometric techniques is based on the principle of producing molecular ions for subsequent separation and analysis. ESI produces ions directly from liquid at atmospheric pressure. For ESI measurements the samples solution are infused into a glass capillary at a constant flow rate and introduced to a "source", where intact ionized molecules in the gas phase are produced. In the mass analyzer the molecular ions are separated on the basis of their mass and charge. Peak intensity in the result referred to the degree of ionization.

Table 4.5.1 Amino acids profile of the Kgalagadi desert truffle

Amino acid	Mw (g)	Intensity
Glycine	74.946	3000
Alanine	89.29	1200
Serine	104.93	250
Proline	114.90	1200
Threonine	119.1	60
Valine	117.15	1200
Cystine	120.93	900
Aspartic acid	132.9	2500
Leusine	131	800
Glu Acid	146.94	100
Lysine	145.92	250
Methionine	149	40
Hystidine	155	37
Phenyl alanine	165.2	60
Arginine	174	17
Tyrosine	181.2	90

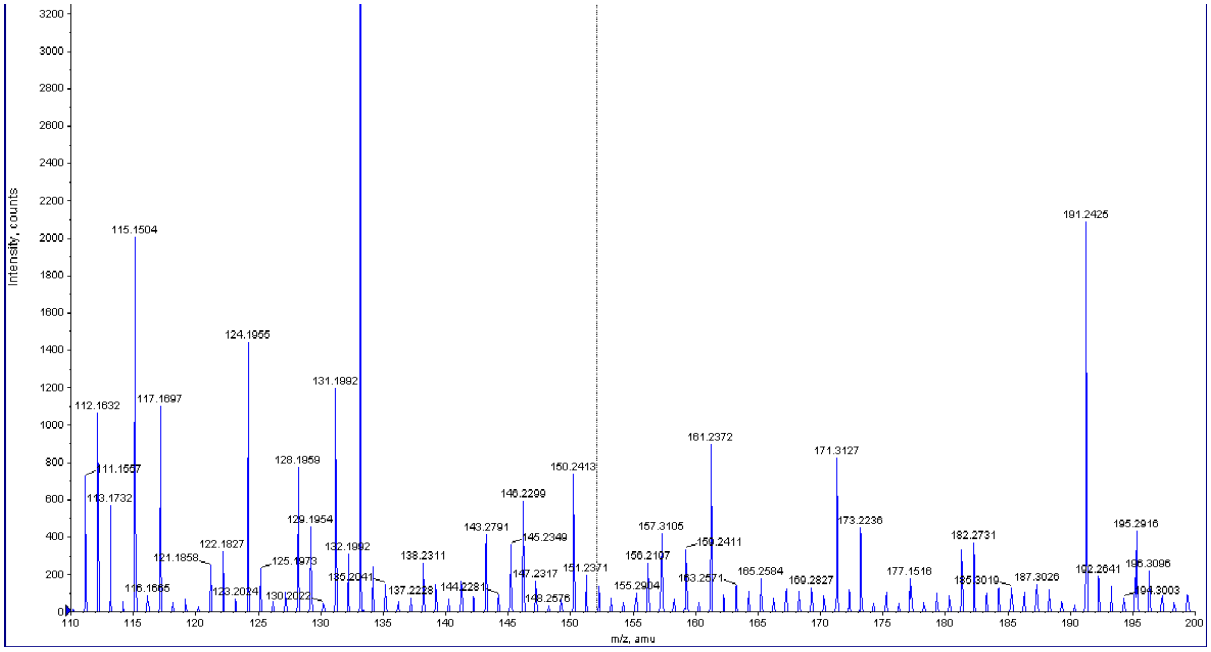


Fig. 4.5.2 (a) Full scan LCMS profiling of fatty acids in truffles

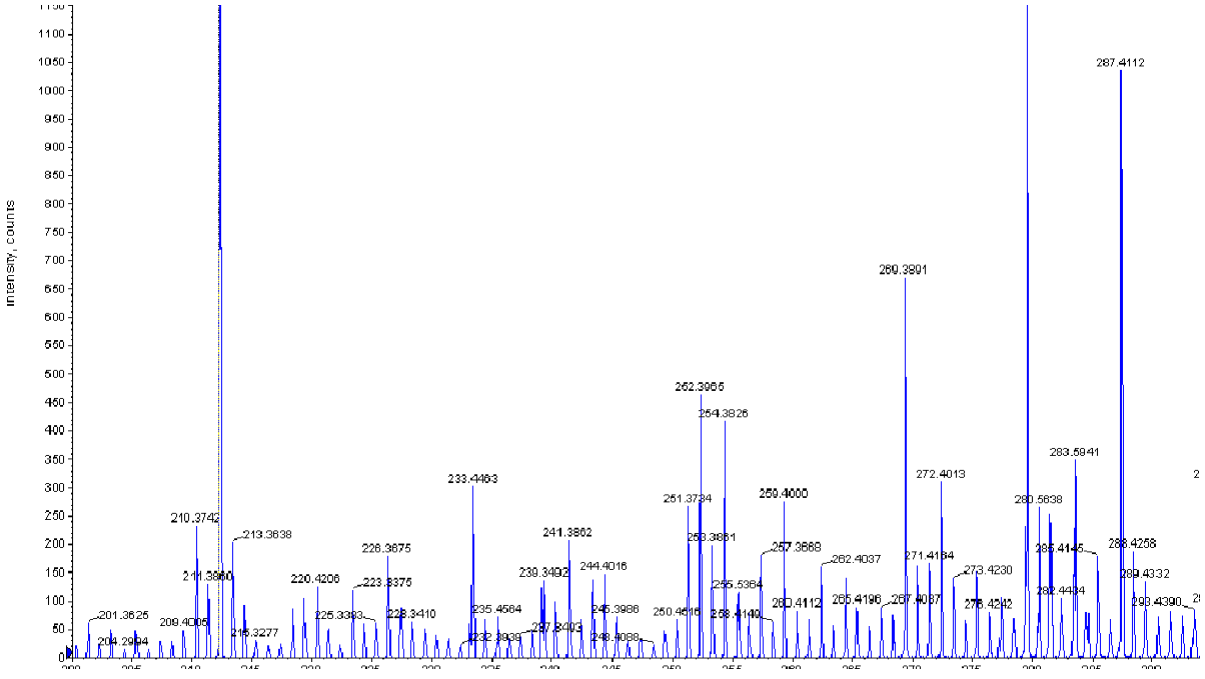


Fig. 4.5.2 (b) Full scan LCMS profiling of fatty acids in truffles

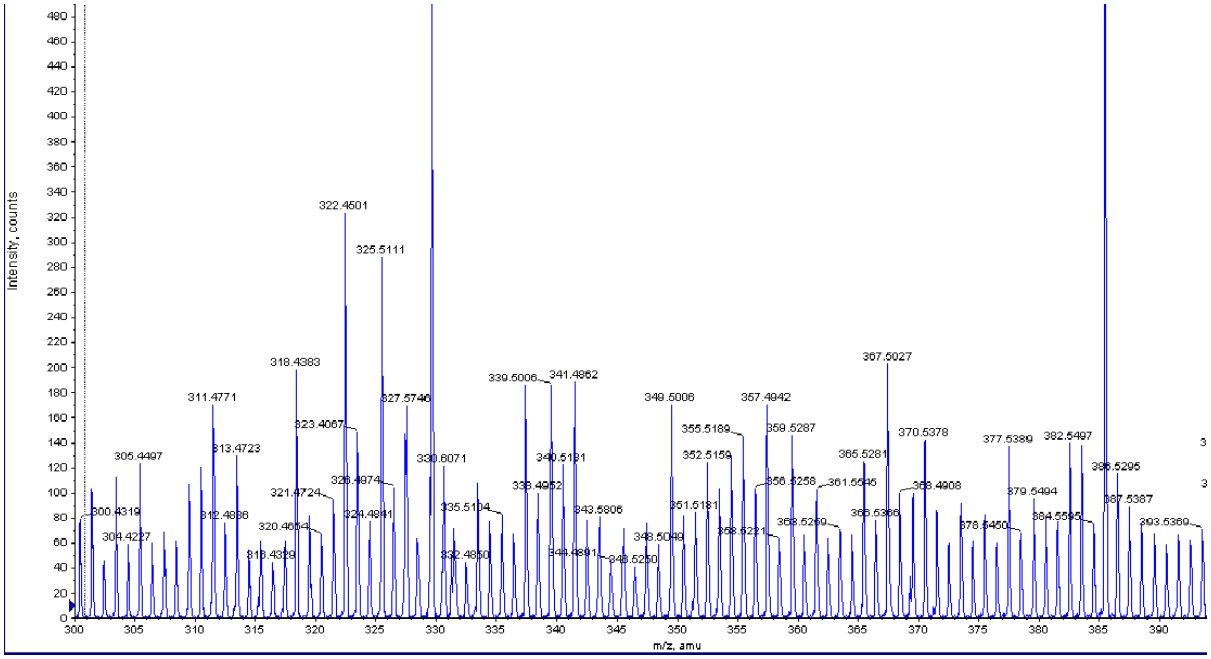


Fig. 4.5.2 (c) Full scan LCMS profiling of fatty acids in truffles

Table 4.5.2 Fatty acids profile of the Kgalagadi desert truffle

Fatty acid	Molecular Mass(g)	Intensity
Caprylic acid	143.27	174.21
Pelargonic Nonanoic acid	157.3	188
Capric acid decanoic	171.3	202
Lauric acid	199.3	230.32
Myristic acid	227.37	258.37
Pentadesilic Pentanedioc	132.12	162.12
Palmitic	255.3	286.42
Palmitoleic	253.38	284.4
Margaric heptadecanoic	269.39	300.45
Stearic Octadecanoic	283.5	314.48
Oleic acid	281.5	312.4
Linoleic acid	279.5	310.45
Linolenic α	277.4	308.43
Linolenic γ	278.43	308.43
Arachidic acid	311.47	342.53
Tricosanoic acid	353.34	384
Lignosteric acid	367.49	398.63

4.3 Analysis of volatile compounds

Gas chromatography is often the technique of choice for separation and analysis of volatile organic compounds. This is because analytes are first transferred into a gas-vapour before instrumental analysis. The analysis of pure volatile compounds is not complicated and can be achieved by direct injection of the analyte into a GC. However, challenges present themselves whenever the analytes of interest are dissolved in a complex matrix such as soil, food, cosmetics, polymers, or pharmaceutical raw materials [11]. GC was used for separation of volatile organic compounds in this study. Extraction techniques employed were used to eliminate possible challenges associated with the matrix, hence the use of GC-HS-SPME.

4.3.1 Selection of best sample matrix for optimal separation of truffle volatile compounds by gas chromatography – head space-solid phase micro extraction (GC-HS-SPME)

The method was developed based on different forms of truffle samples to evaluate sensitivity. Optimization results indicated that fresh truffle slices were the best samples to use for HS-SPME-GCMS volatile compound analysis. There was good separation for all compounds between different fibers. PDMS 100 µm SPME fiber was used as the invariable factor to keep the fiber type constant and vary the matrix. The fiber showed the ability to adsorb volatile compounds associated with the Kalahari Dessert truffle. This headspace technique gave five neatly separated peaks (Fig 4.6.1 (C)) for truffle slices. Truffle liquid and paste only managed to separate two compounds each. The compounds were only evaluated for the degree of separation. The peak identities could not be given at this stage standards of known peak areas were not yet included in the study.

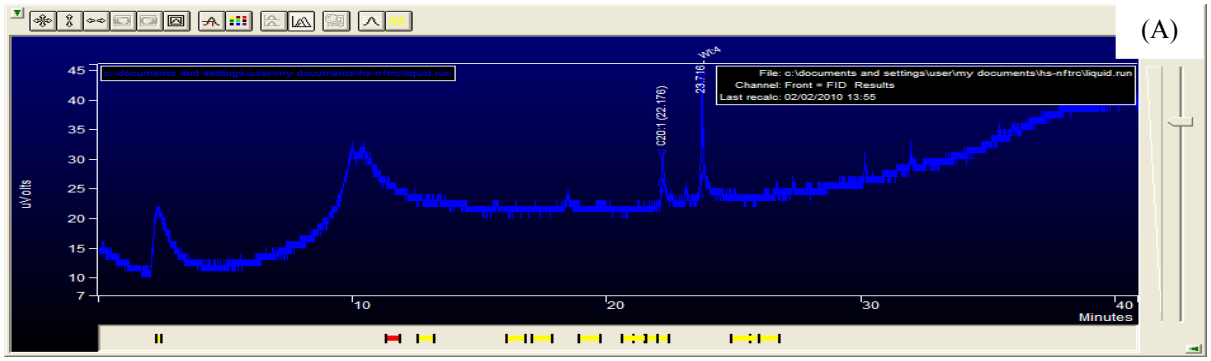


Fig. 4.6.1 A GC-FID chromatogram of volatile compounds from Kgalagadi desert truffle liquid by HS-SPME PDMS 100 µm fiber;, CP – Sil 30 m x 0.32 mm x 0.25 µm column

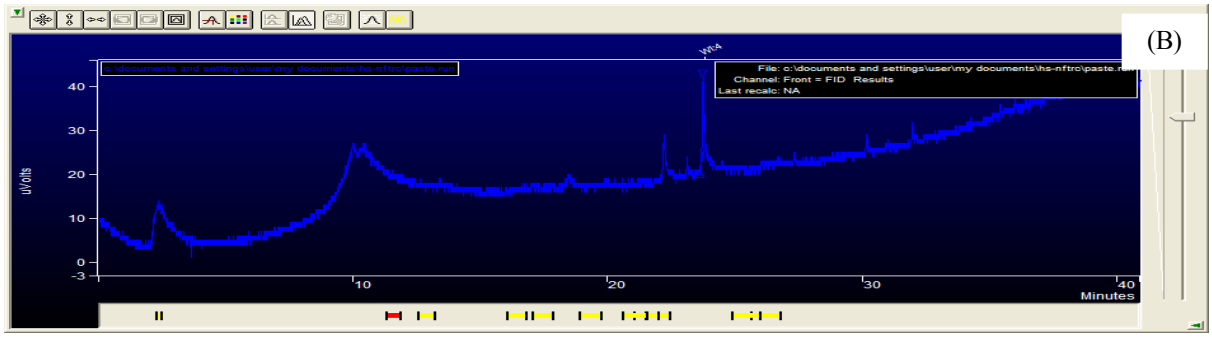


Fig. 4.6.1 B GC-FID chromatogram of volatile compounds from Kgalagadi desert truffle paste by HS-SPME PDMS 100 µm fiber; CP – Sil 30 m x 0.32 mm x 0.25 µm column

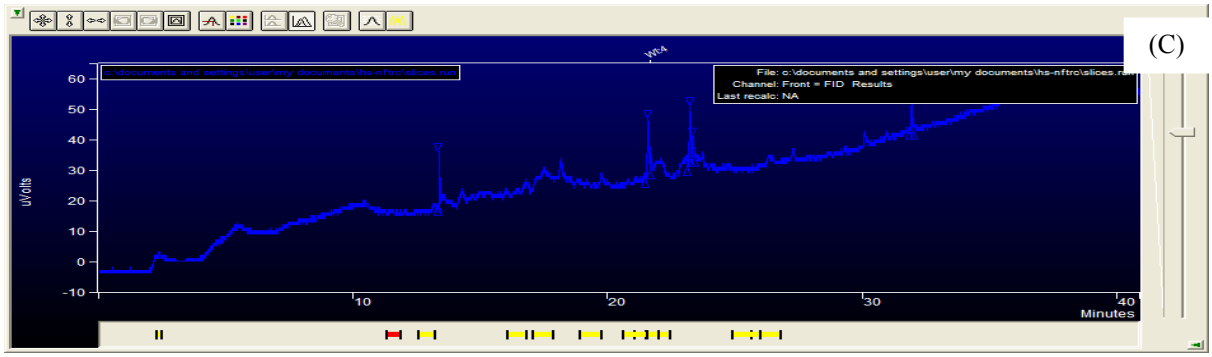


Fig. 4.6.1 C GC-FID chromatogram of volatile compounds from Kgalagadi desert truffle slice by HS-SPME PDMS 100 µm fiber; CP – Sil 30 m x 0.32 mm x 0.25 µm column.

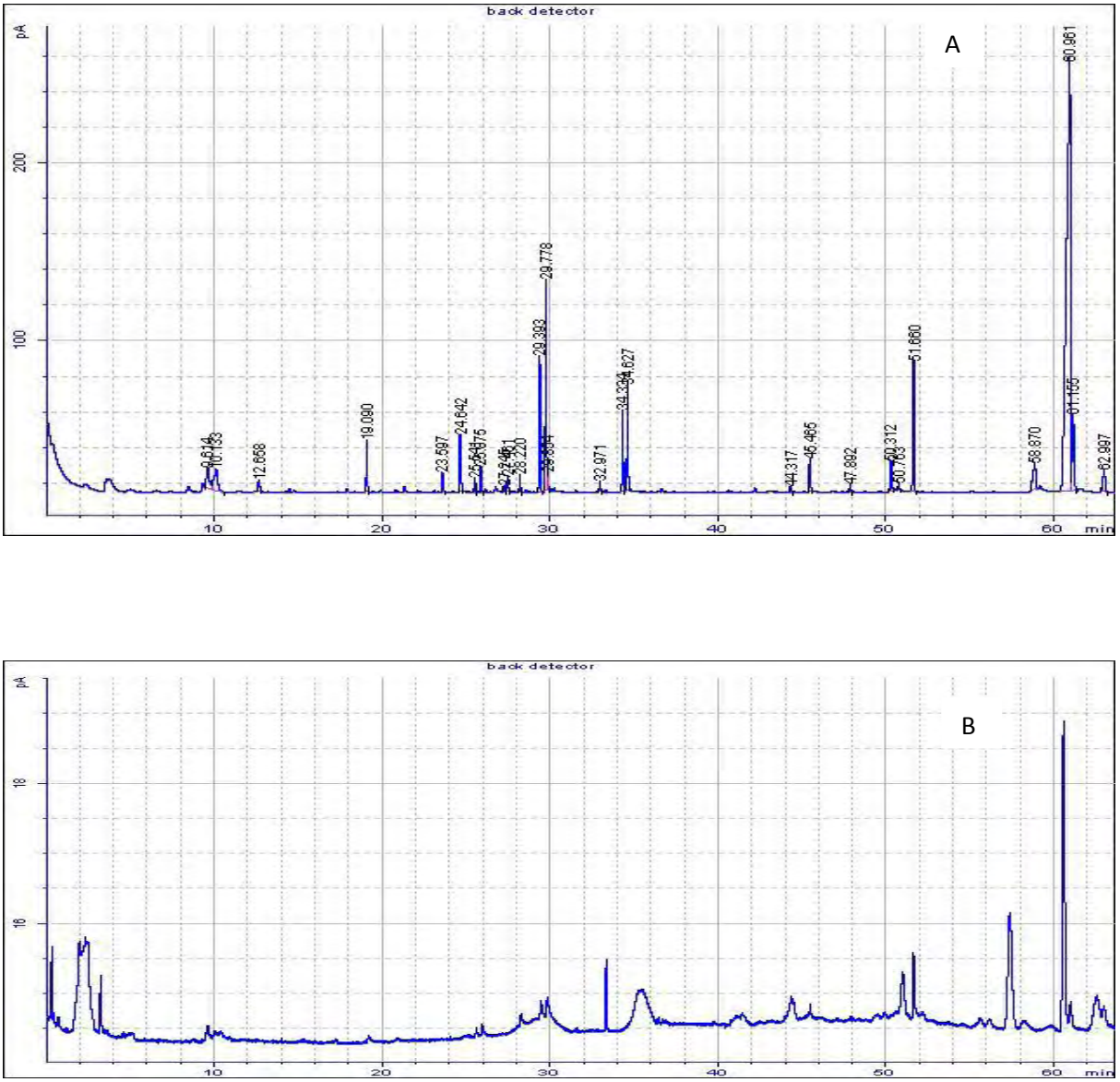


Fig. 4.6.2 GC-FID chromatograms of volatile compounds from Kgalagadi desert truffles (A): PDMS 100 µm truffle slices (B): PDMS 100 µm truffle liquid sample chromatographic column; ZB - 30 m x 0.25 mm x 0.25 µm method optimisation.

The optimization process showed that liquid extracts of truffles did not contain a lot of volatile compounds. The sampling process was conducted in the same way as of solid samples using SPME. The result set optimum conditions required for future work involving the best extraction matrix for use in profiling for volatile compounds in truffles.

4.3.2 Best fiber selection for extraction of truffles for qualitative analysis of volatile organic compounds using GC, HS-SPME procedure

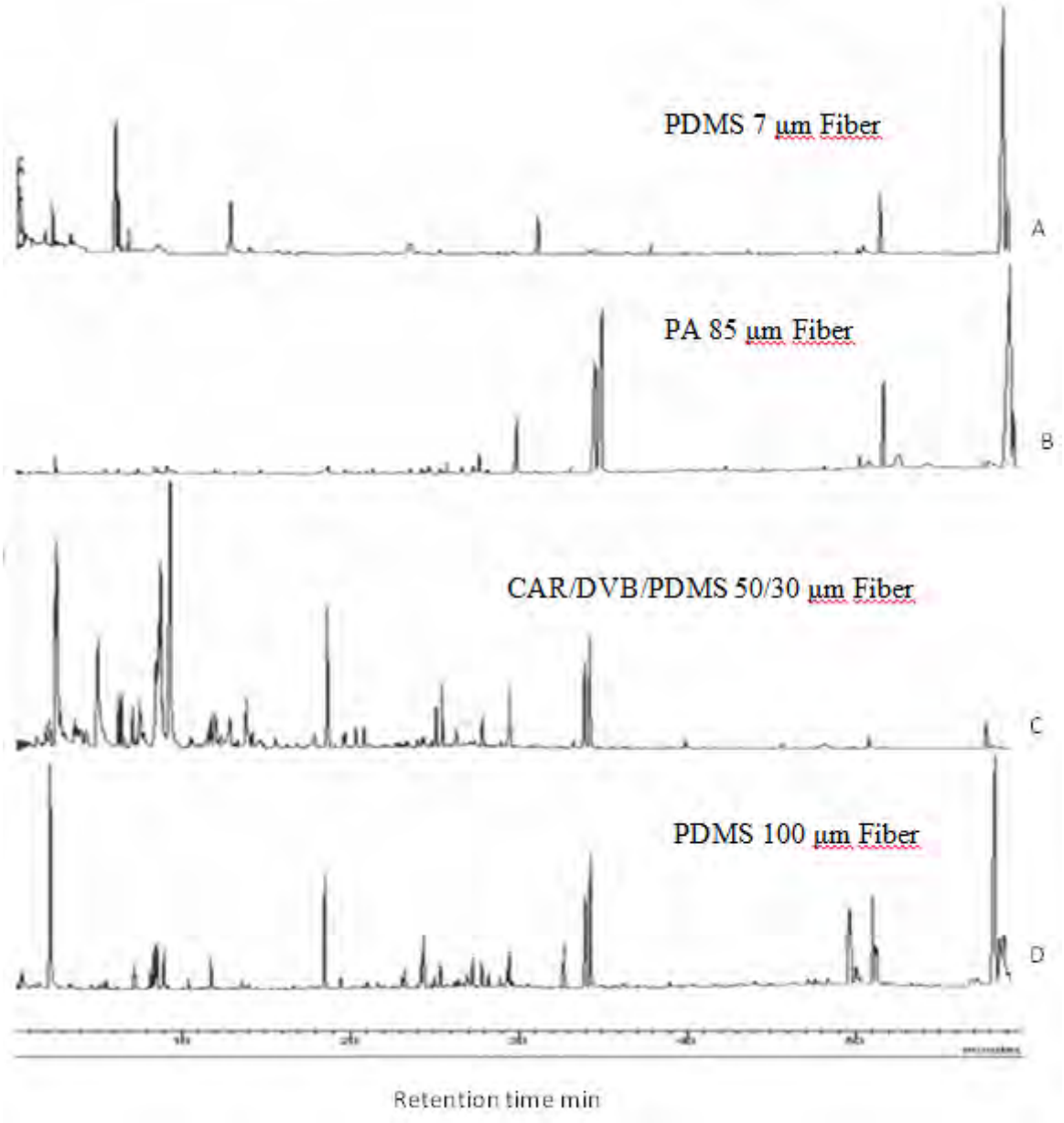


Fig. 4.6.3 GCMS chromatograms of volatile compounds from Kgalagadi desert truffle for four fibres; A: PDMS 7 µm , B: PA 85 µm, C:CAR/DVB/PDMS 50/30 µm, D: PDMS 100 µm, GC column; VF 5ms 30m x 0.25 mm x 0.25 µm.

Figure 4.6.3 above compares GC responses of four different fibers. More peaks were observed from the PDMS 100 um extract as compared to the other three fibers after a run time of an hour. Therefore PDMS 100 um fiber was selected for extraction since it gave better GC separation of compounds identification of volatile compounds using GC-MS. A constant sample matrix of truffle slices was used under the same GC conditions.

The best SPME fiber for an effective extraction and subsequent desorption and separation of volatile compounds was discovered to be PDMS 100 um. The peaks separated from this extraction were hence assigned peak identities (Fig 4.6.4). Subsequent work involving compound identification was then based on these conditions that gave the best separation. Hence the focus of the research from this stage onwards discuses only volatile organic compounds extracted by PDMS 100 um SPME fiber and separated by GC with FID.

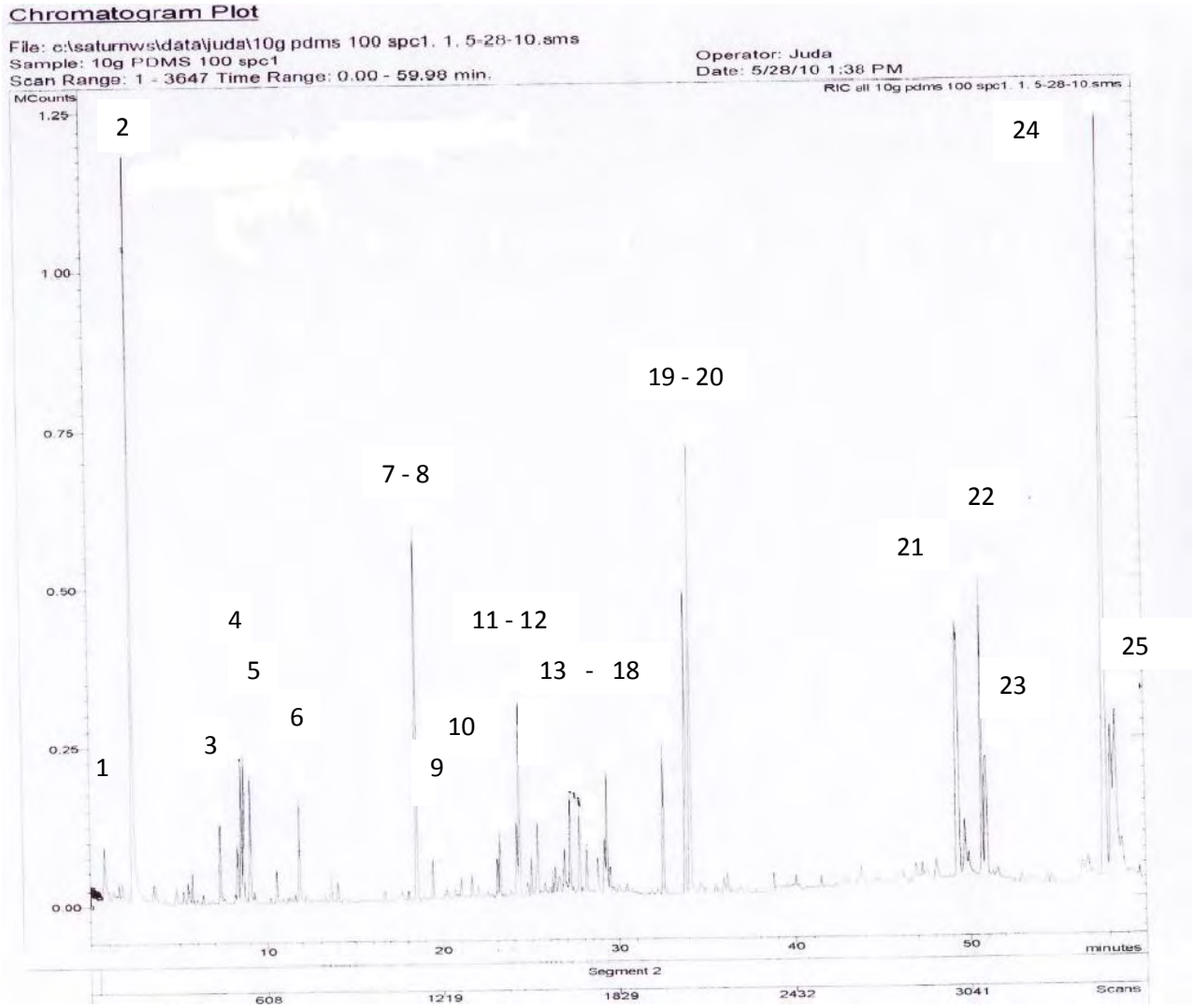


Fig. 4.6.4 Peaks separated by GC from desert truffle extracts using PDMS 100 um SPME fibre

Chromatogram Information was obtained for the 25 peaks separated from the truffles using PDMS 100 um SPME fiber for extraction. The data was recorded on Table 4.6.4 below.

Table 4.6.4 A summary of peaks extracted by PDMS 100 um SPME fiber

Peak Number	Retention Time	Peak Area
1	0.777	370797
2	2.410	5031500
3	7.342	490724
4	8.677	917111
5	9.089	922609
6	11.860	646208
7	18.521	2342536
8	19.461	226905
9	23.213	404144
10	24.341	1289797
11	24.982	257836
12	25.337	581554
13	27.202	584325
14	27.737	515673
15	28.134	256467
16	29.347	755760
17	32.553	987932
18	33.808	2281961
19	34.150	3664405
20	49.288	2345956
21	50.682	1960538
22	50.898	1134614
23	57.855	11062002
24	58.136	2127315
25	58.400	3452506

The analysis of volatile organic compounds by HS-SPME is relatively straight forward but care needs be taken to avoid sample carryovers hence false positives. Carry overs should be avoided by running blank SPME fibers to desorb all the analytes before resampling. However some researchers prefer to use fresh fibers instead of fiber reconditioning prior to sampling [71]. All SPME fibers used in this study were preconditioned according to manufacturer's instructions prior to use.

4.3.3 Identification of compounds

PDMS 100 um was selected for use as the best fiber for the analysis of volatile compounds. GC-was used for separation and electron ionization mass spectrometry (EI-MS) fragmentation was employed for positive identification. The advantage of EI-MS is that fragmentation is extensive, giving rise to a pattern of fragment ions which can help to characterize the compound [73]. Compounds identities were confirmed using the saturn library search software as per the example in Figure 4.6.5 below. The use of EI-MS together with the saturn library search gave reliable fingerprint identities of the compounds.

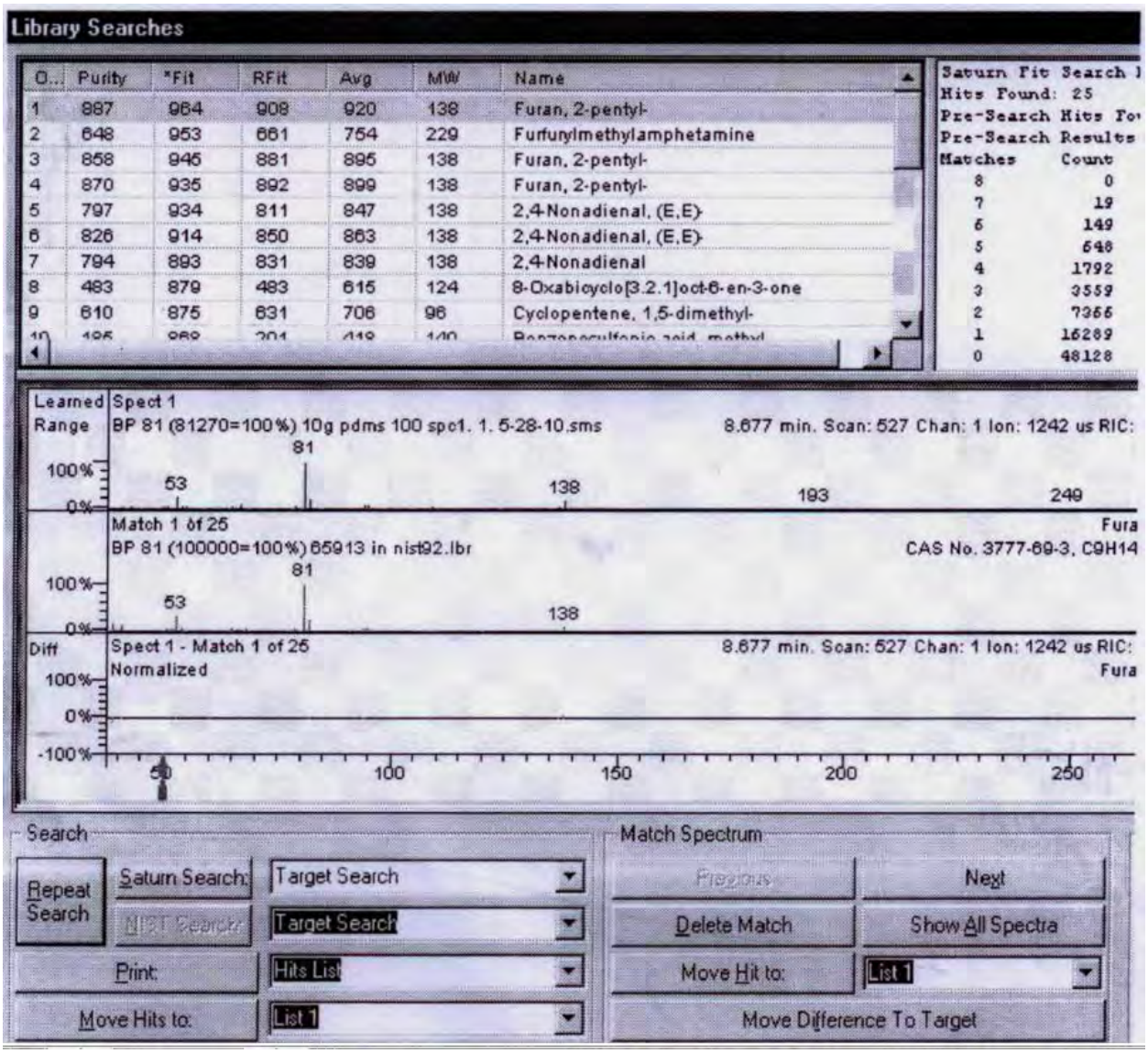


Fig. 4.6.5 Compound identification by saturn library search software

The output (Fig.4.6.5) above depicts a library search report after steps taken to identify peak number four in table 4.6.4 above. The Retention time of the compound during separation was

8.677 minutes and the peak area was 917111. In order to identify this compound from the chromatogram, click on the GC-chromatogram, that is the FID signal.

Since the GC was coupled to the Varian 2000 mass spectrometer, selecting a peak from GC separation chromatograms would give its EI-MS fragmentation report. The fragments of the compound at peak four are produced as an effect of bombardment during Electron Ionisation.

The Saturn inbuilt GCMS library search software was used to show all possible combination products of the fragmented compound. The best match for the compound description is done by studying the MS Chromatographs and comparing them with those available in the library. In the example of peak four above there were 25 possible products with all their names listed on the top left table of figure 4.6.5.

The selection of the best identity of the compound was done by using information provided by the table. In the example given, a compound of highest purity level of 997 and the highest Fit of 964 was selected. The information translated into 99.7% purity of the product and 96.4% chances of it being that. In this case the product was identified as Furan, 2-pentyl ($C_9H_{14}O$) with a molecular weight of 138 g, a known flavor compound in natural products.

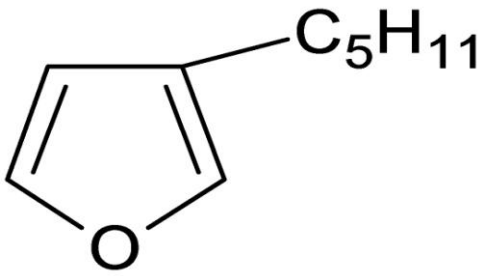


Fig. 4.6.6 Structure of Furan, 2-pentyl ($C_9H_{14}O$)

The compound identity protocol described above was applied to all GC separation chromatograms described on table 4.6.4 A summary of peaks extracted by PDMS 100 um SPME fiber. After their identities were confirmed by the Saturn library search a report detailing a profile of compounds in the Kgalagadi desert truffle was produced (see Table 4.6.5).

The same procedure was done for the other SPME Fibers (PDMS 7 µm, PA 85 µm, and CAR/DVB/PDMS 50/30 µm,) to identify compounds they extracted which were successfully

separated by GC. This information was used to help select the best fiber for the study only hence it does not form part of this discussion. The information is as displayed on Appendix 3.

The challenge in this procedure was mainly due to the use of a limited GCMS library. Identified compounds are listed in the table below.

Table 4.6.5 Volatile compounds identified in Kgalagadi dessert truffles after sampling with a PDMS 100 µm SPME fiber

Peak No	Compound Name	Mol. Weight	Purity	Fit	Retention time	Area
1	1-Acetoxy-2-(dodecyloxy)ethane	272	449	710	0.777	370797
2	2-t-butyl-2,3-dimethyl-3-buten-1-ol	156	218	904	2.410	5031500
3	2-heptenal,(z)	112	788	910	7.342	490724
4	Furan,2-pentyl	138	887	964	8.677	917111
5	hexanoic acid,ethyl ester	144	724	862	9.089	922609
6	9-oxabicyclo[6.1.0]nonane	126	675	903	11.860	646208
7	octanoic acid,ethyl ester	172	611	876	18.521	2342536
8	1,4-hexadiene,3-ethyl	110	747	884	19.461	226905
9	2,4-decadienal,(E,E)	152	764	904	23.213	404144
10	phenol,3-methyl-6-propyl	150	779	842	24.341	1289797
11	2,4-decadienal,	152	753	941	24.982	257836
12	5,-isopropyl-8-methyl-5,6,7,8 tetra hydro-2,4-quinazolinedione	222	418	948	25.337	581554
13					27.202	584325
14	Ethyl palmitate	284	560	909	27.737	515673
15	ethyl 7-oxa-7-(2,2-dimethylcyclopentyl) heptonoate	256	282	819	28.134	256467
16	adamantamone	150	529	871	29.347	755760
17	azelaaldehydic acid, butyl ester	228	441	788	32.553	987932
18	disulfide,ethyl hexyl	164	340	865	33.808	2281961
19	Benzoic acid, 2-4-dihydroxy-3,6-dimethyl-methyl ester	196	632	799	34.150	3664405
20	dibutyl phthalate	278	784	938	49.288	2345956
21	hexadecanoic acid,ethyl ester	284	607	952	50.682	1960538
22	diazene,bis(4-methoxyphenyl	242	482	902	50.898	1134614
23	9,12-octadecanoic acid (2,2)-,2,3-dihydroxypropyl ester	354	693	944	57.855	11062002
24					58.136	2127315
25	octadecanoic acid	284	495	966	58.400	3452506
	Totals					**44611175**

PDMS 100 µm SPME fiber coating is widely used for volatile compounds by headspace. In this study, the fiber trapped the most number of volatile compounds from the truffles. A total of 24 volatile compounds were identified with a molecular weight range of 110 – 354, the most prominent peak being that of 2-t-Butyl-2,3-dimethyl-3-buten-1-ol ($C_{10}H_{20}O$, MW 156).

PDMS 100 µm extracted 24 compounds being the only fiber in this study that showed the first sulphur compound in the truffle volatiles series (disulphide, ethyl hexyl benzoic acid, 2-4-dihydroxy-3, 6-dimethyl-methyl ester). This compound was separated as peak number 18 with a retention time of 33.808 minutes.

Studies on truffles show that most of the aroma compounds are based on sulphur based compounds [8]. The Ehrlic system (Fig 2.1.1) outlined in this thesis states pathways leading to characteristic truffle volatile compounds. It also proves the fact that formation of sulphur compounds in truffles occurs as a result of a chemical non-enzymatic degradation of 3-(methylthio) propanal.

Although the source of aroma compounds in truffles is mostly known to be alcohols and sulphur (see Fig 2.1.1 and Fig 2.1.2) based compounds [8, 53, and 71], in this study the identified compounds were mostly fatty acid methyl esters. The result is also expected because alcohols contained in truffles are a product of biosynthetic process of enzymes. This involves oxidation of fatty acids and the process of cleaving to produce short chain volatile compounds [71]. This result could be attributed to loss of certain classes of volatiles due to the length of storage and sample handling before analysis. Due to the difficulty associated with sourcing dessert truffles, samples can be kept for long periods before research is carried out, to this end; development of optimum analytical protocols is recommended for prompt analytical work on truffles.

Chapter 5

Conclusion and Recommendations

Truffle morphology was studied successfully. Truffles were found to have spores of about 2.5 µm situated mostly at the heart of the fruiting body. The SEM studies showed that truffles had a gummy substance that was presumed to prevent observation under the microscope unless treated by hexamethyldisilazane ($C_6H_{19}NSi_2$).

Extracts of truffles were successfully obtained employing the tuneable dynamic PHWE technique within the temperature range of 60 - 120 °C, and thus facilitated the identification of functional groups by FTIR.

Specific FTIR spectral regions were identified which could be useful in monitoring the quality of truffle oil. The information provided by FTIR spectra allowed us to identify most of the chemical constituents of the Kgalagadi desert truffles. The main constituents of truffles were concluded to be proteins and polysaccharides. The developed method is recommended for use in monitoring differences in spectra of truffles for; discrimination of different species, discrimination of same species from different sources, degree of oxidation of oil and evaluation of the state of health of truffles. It is also recommended for use in adulteration monitoring in truffle oil by studying band shifts especially at 3009 cm^{-1}. Although the spectra resembled that of typical edible oil, further work is recommended so as to identify specific fatty acids spectra using known standards since the study was based on whole truffle oil. The FTIR spectroscopic method was very useful in truffle studies as a non-destructive and rapid analysis tool.

Truffles were shown to contain both essential and non-essential amino acids. Further work on quantification of the amino acids with known standards in the desert truffles is recommended.

The majority of the fatty acids identified exhibited saturated structures. Further work on quantification of the fatty acids with known standards in the desert truffles is recommended.

The application of SFE was unsuccessful. Due to low extraction yields, truffle volatile extracts from SFE gave extraction yields much lower than the detection limits for GC-FID or GCMS. The low yields were attributed to the loss of volatile compounds during sample

preparation. While insufficient extraction time may also be the reason for poor yields, it was also suspected that the sample matrix was not compatible with SFE characteristics. The recommendation for future use of SFE in truffle volatile work is to further optimize extraction conditions so as to improve the method's efficiency.

Volatile constituents of the Kgalagadi desert truffle were successfully profiled employing SPME equipped with a PDMS 100 μm fiber for extraction. Fresh slices from the heart of truffles were the best samples to use for HS-SPME-GCMS volatile compound analysis. The characteristic volatile profile of the Kgalagadi desert truffle contained a total of 24 compounds. As per literature, sulphur and alcohol compounds associated with truffle aroma were identified within the profile. The most prominent peaks identified in the profile belonged to the following group of compounds; 2-t-butyl-2,3-dimethyl-3-buten-1-ol, disulfide, ethyl benzoic acid 2-4-dhydroxy-3,6-dimethyl-methyl ester, 8,11-octadecanoic acid methyl ester, benzoic acid, 2,4-dihydroxy-3,6-dimethyl-methyl ester, isoquinoline, 1 butyl-3,4-dihydro and 3-heptanone, 6 methyl. It is recommended that further work should be done to quantify these volatile compounds including quantification of fatty acids methyl esters since the volatile profile of the Kgalagadi desert truffle contained mainly fatty acid methyl esters and unsaturated aliphatic hydrocarbons.

In conclusion, green technology based sample preparation techniques were used successfully to give a profile of volatile compounds of the Kgalagadi desert truffle. Two extraction techniques of SPME and SFE were successfully evaluated for the extraction of volatile compounds.

Finally, it is recommended that more work be done to further quantify the Kgalagadi desert truffle volatile compounds using known standards. Future studies should include lipid classes and research on the claimed neutraceutical properties of the Kgalagadi desert truffle. It is recommended that PHWE extracts be further evaluated for compounds of neutraceutical importance like phytochemicals and antioxidants. This would add value on defining and classifying the functional components of truffles and other foods indigenous to Botswana. The indigenous knowledge system of the local consumers could be enhanced by identifying and quantifying truffle medicinal extracts through exploring these sustainable green technology protocols of SFE and PHWE further. This study adds to the already existing limited information published on the Kgalagadi desert truffle hence it forms a sound basis for further research.

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APPENDIX

Appendix 1.1 - 1.2 Shows Fiber Conditioning Protocols used as Per Manufacturer's Instructions

Appendix 1.1 SPME Fiber Conditioning Protocols used as per Manufacturer's Instructions

SPME Fiber	Conditioning Temperature in °C	Time of exposure
PDMS 100 µm	250	30 min
PDMS 7 µm	320	60 min
Polyacrylate 85 µm	280	60 min
DVB/CAR/PDMS 50/30 µm	270	60 min

Appendix 1.2 Shows Cleaning Protocols used on SPME Fibers

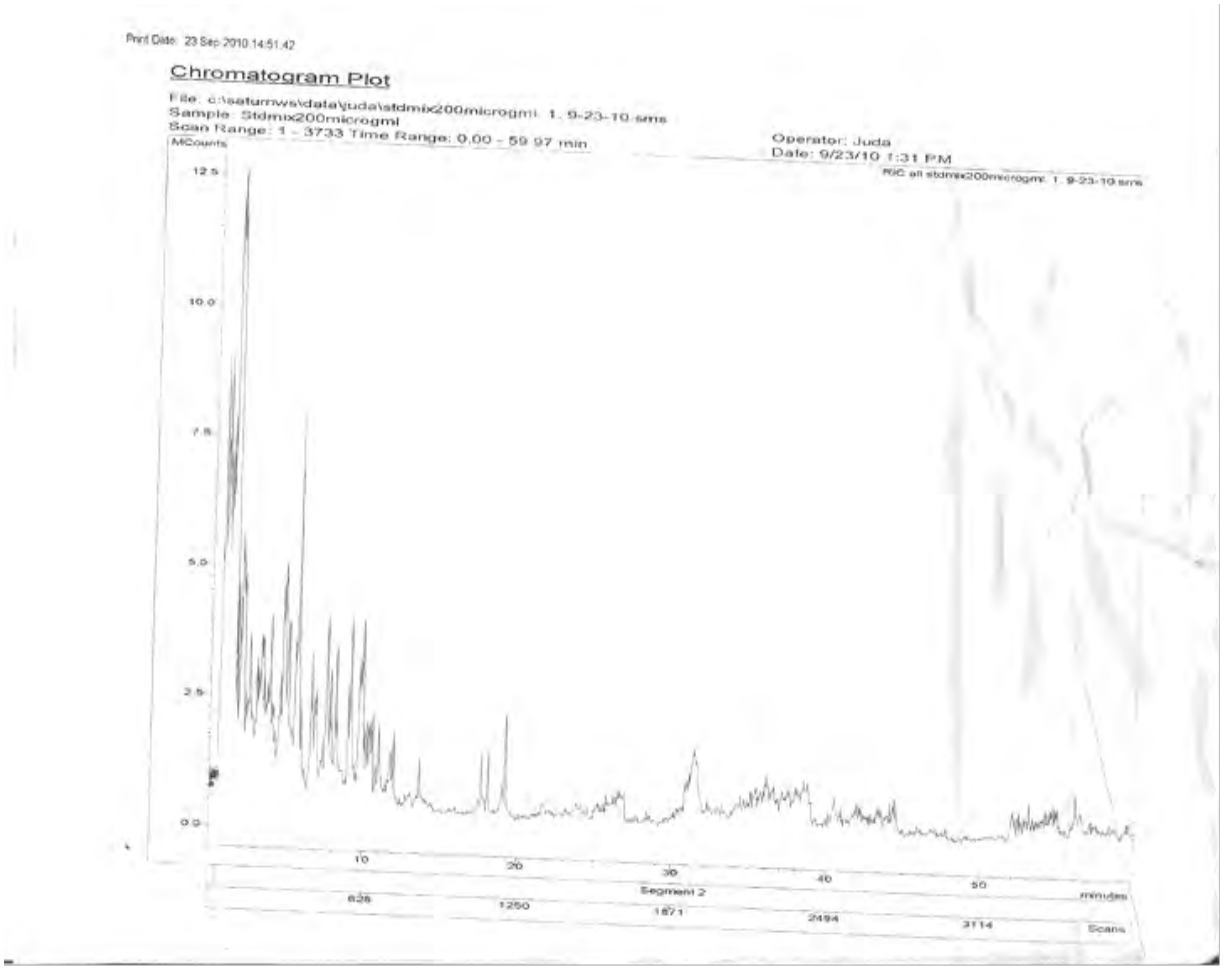
SPME Fiber	Cleaning Solution	Time of exposure
PDMS 100 µm	50 : 50 Water : Ethanol	30 min
PDMS 7 µm	50 : 50 Water : Ethanol	30 min
Polyacrylate 85 µm	Ethanol	30 min
DVB/CAR/PDMS 50/30 µm	50 : 50 Water : Ethanol	30 min

*After 30 min cleaning fibers should be rinsed in ultrapure water and placed on a clean filter paper to air dry.

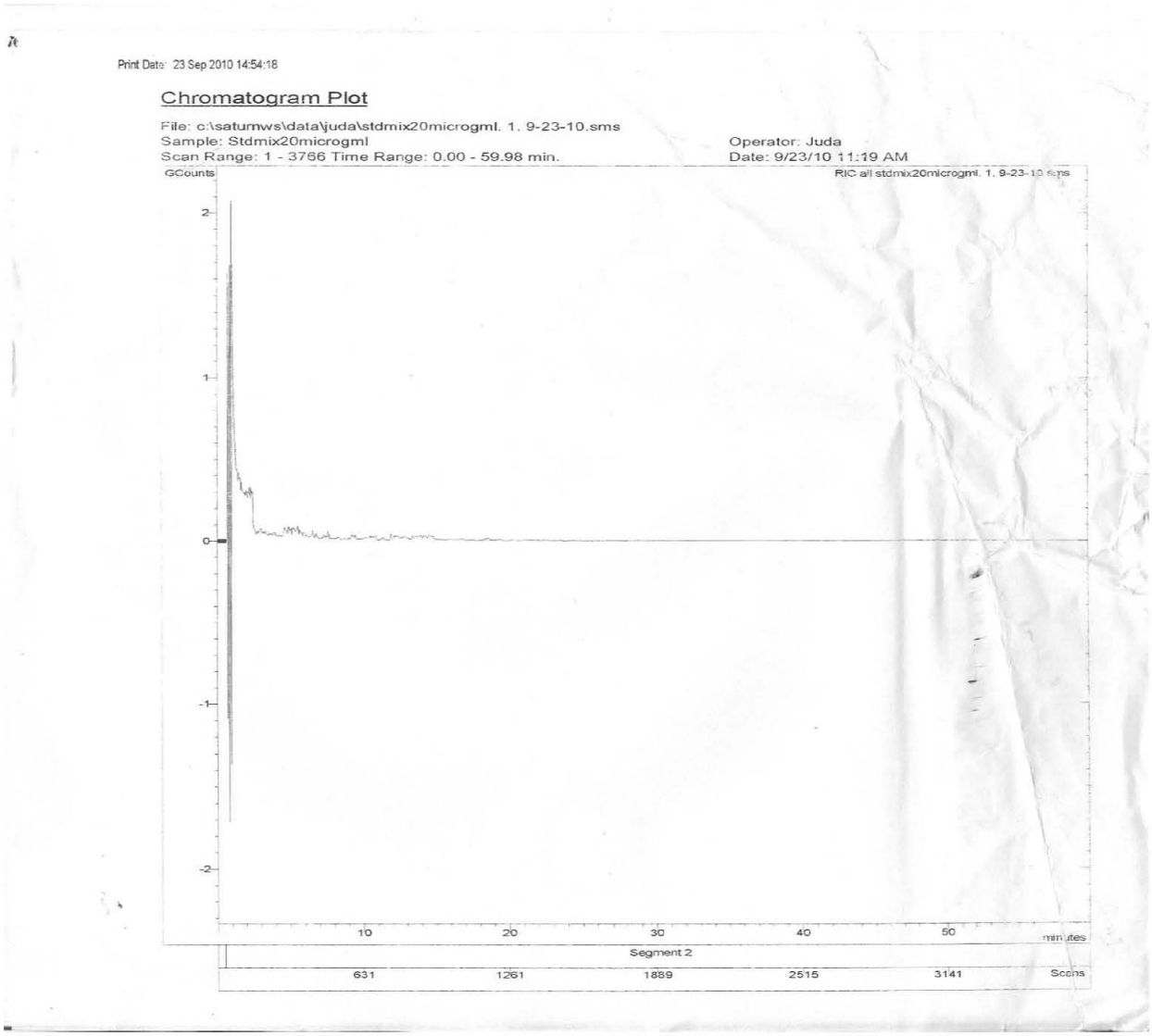
Appendix 2.1 - 2.9 shows evaluation of truffle volatiles by SFE-GCMS and SFE coupled to

HS-SPME-GCMS

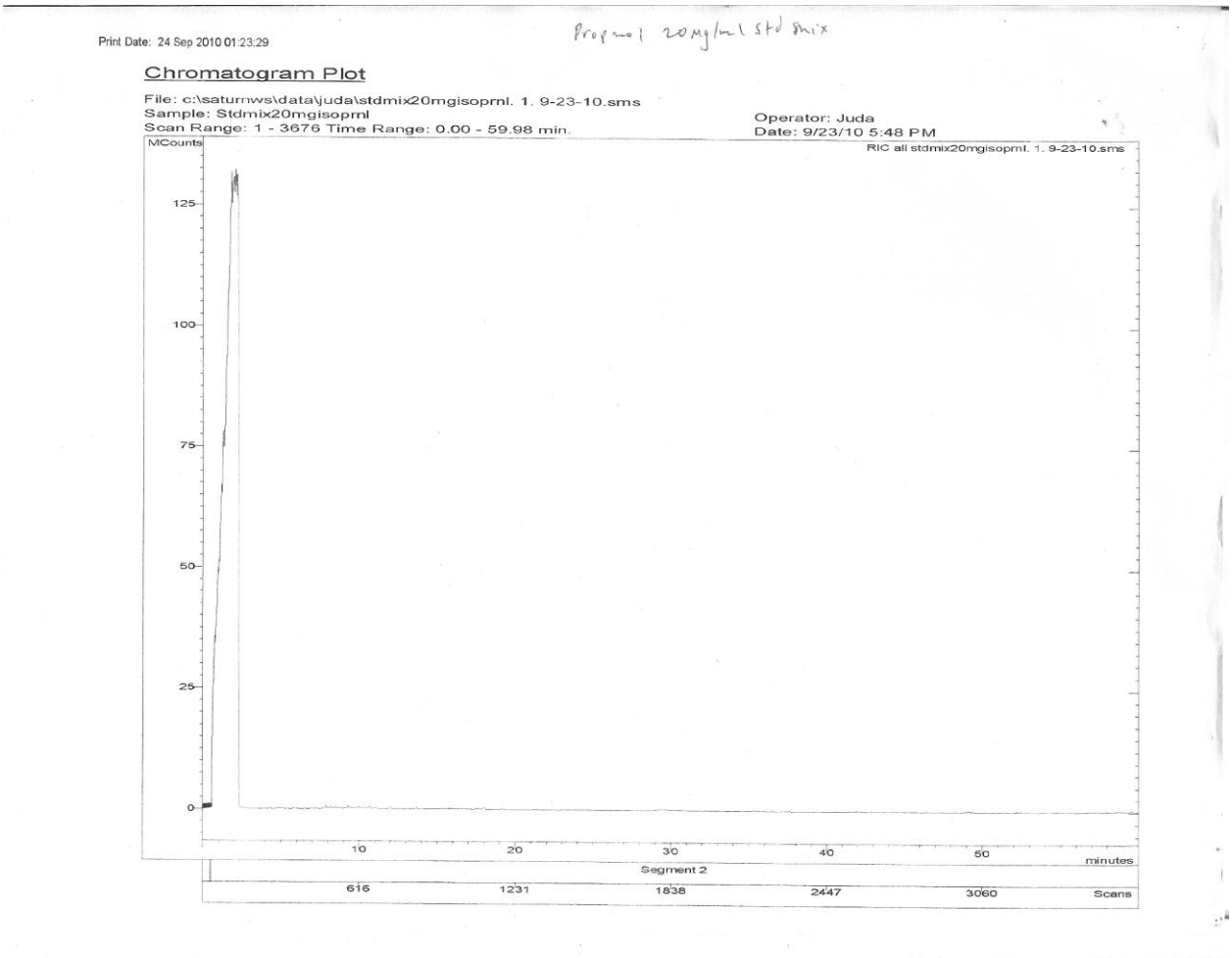
Appendix 2.1 GC Separation of an EPA 524 VOC Mix Calibration standard of Concentration 200 µg / ml in propanol. GC-FID, column; VF 5ms 30m x 0.25 mm x 0.25 µm



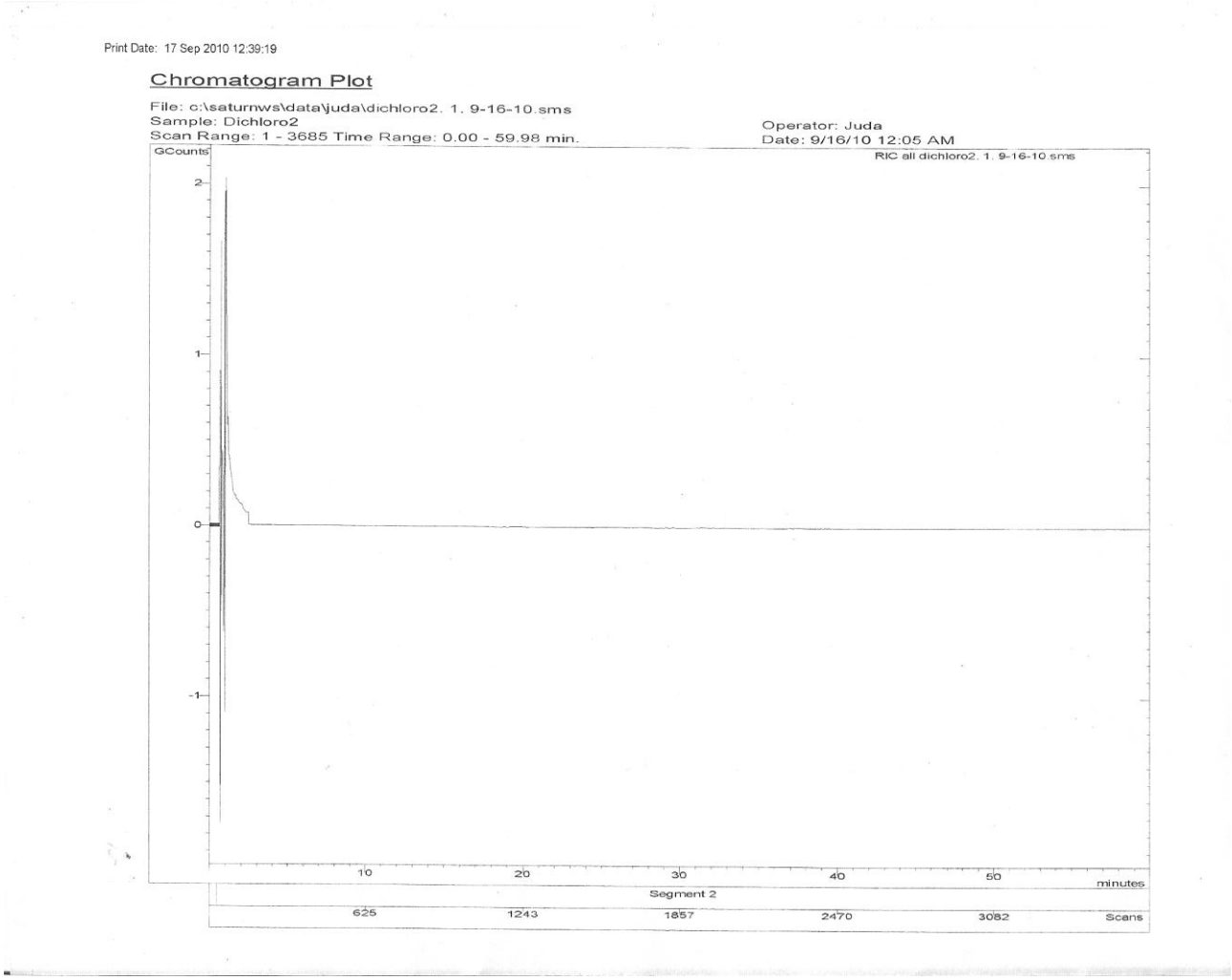
Appendix 2.2 GC Separation of an EPA 524 VOC Mix A Calibration standard of Concentration 20 µg / ml in propanol GC-FID, column; VF 5ms 30m x 0.25 mm x 0.25 µm



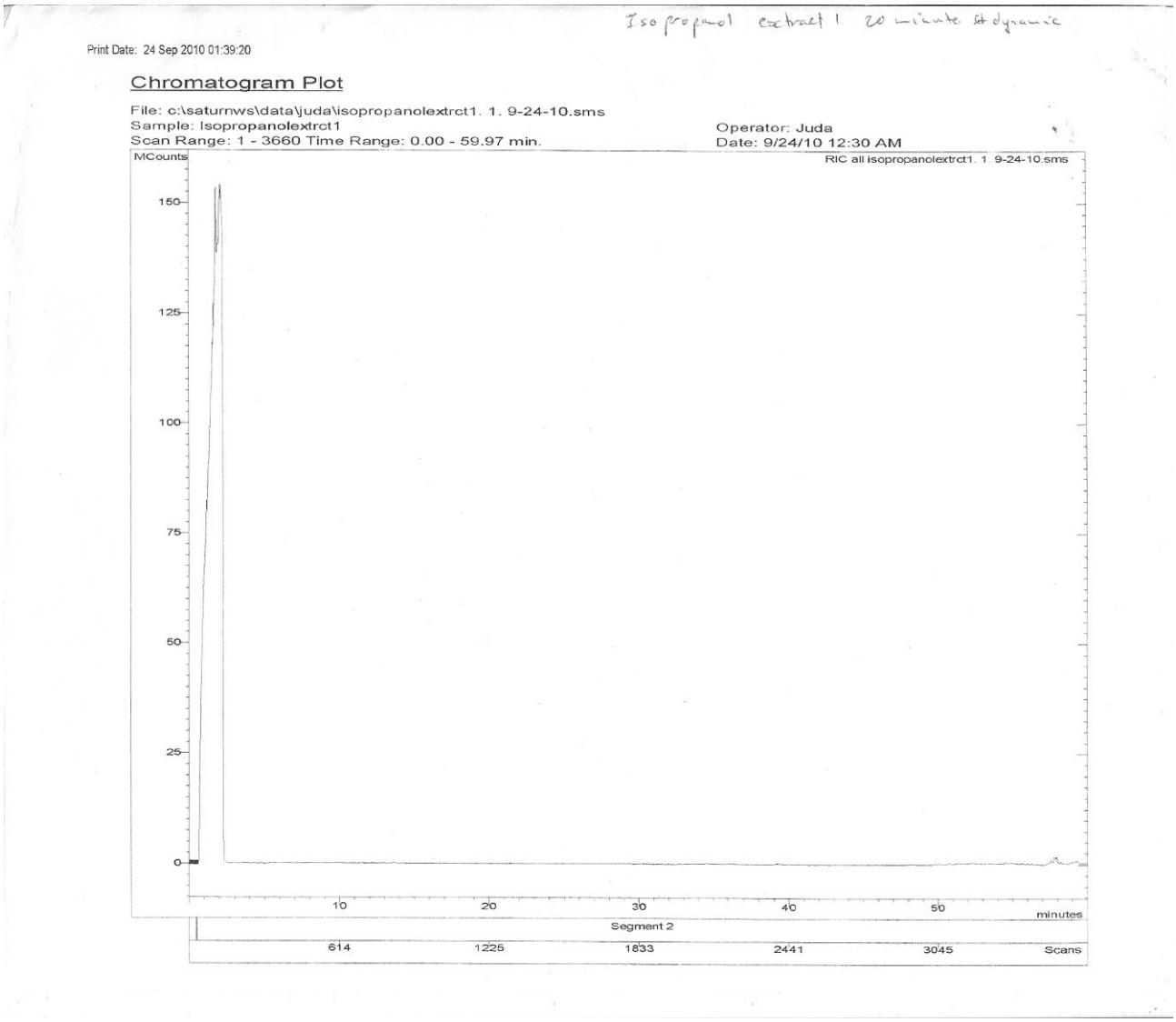
Appendix 2.3 GC Separation of an EPA 524 VOC Mix A Calibration standard of Concentration 20 µg / ml in propanol GC-FID, column; VF 5ms 30m x 0.25 mm x 0.25 µm



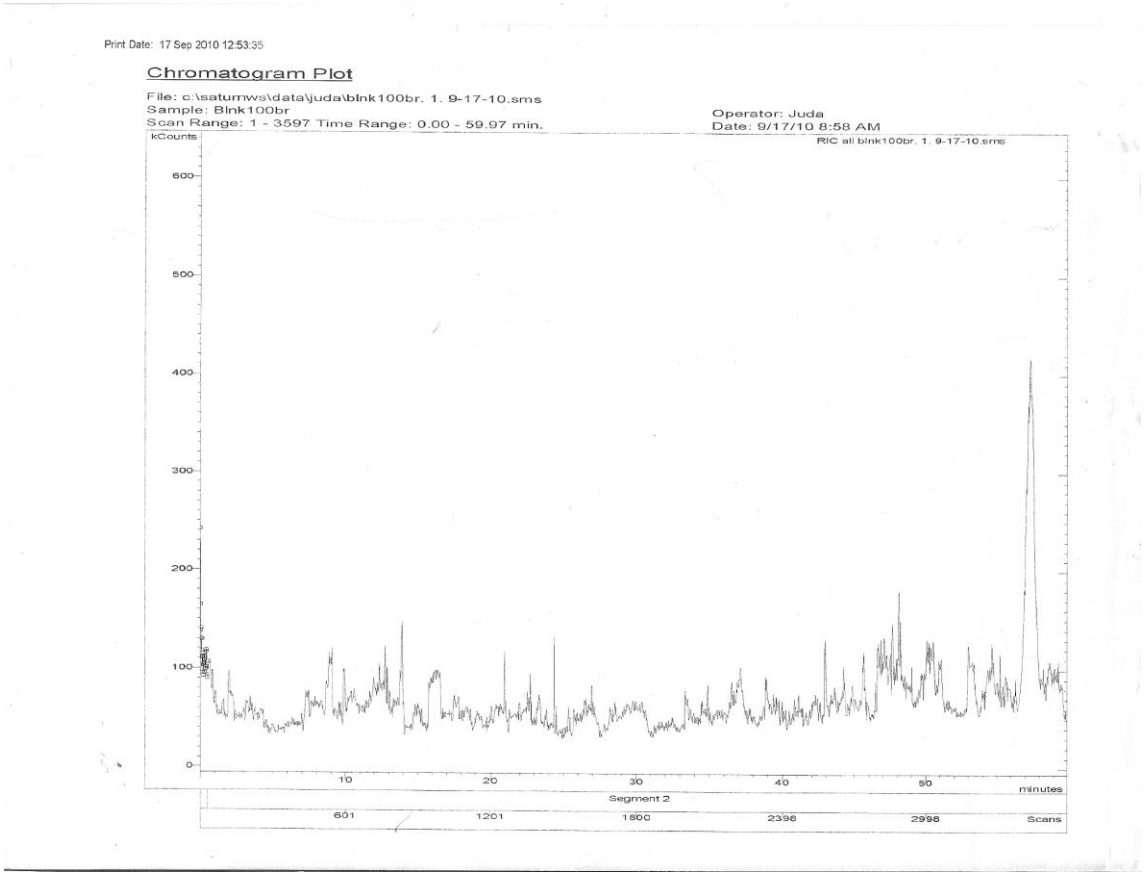
Appendix 2.4 GC Separation of an SFE Truffle extract collected in dichroloromethane after 20 minutes of dynamic extraction. GC-FID, column; VF 5ms 30m x 0.25 mm x 0.25 µm



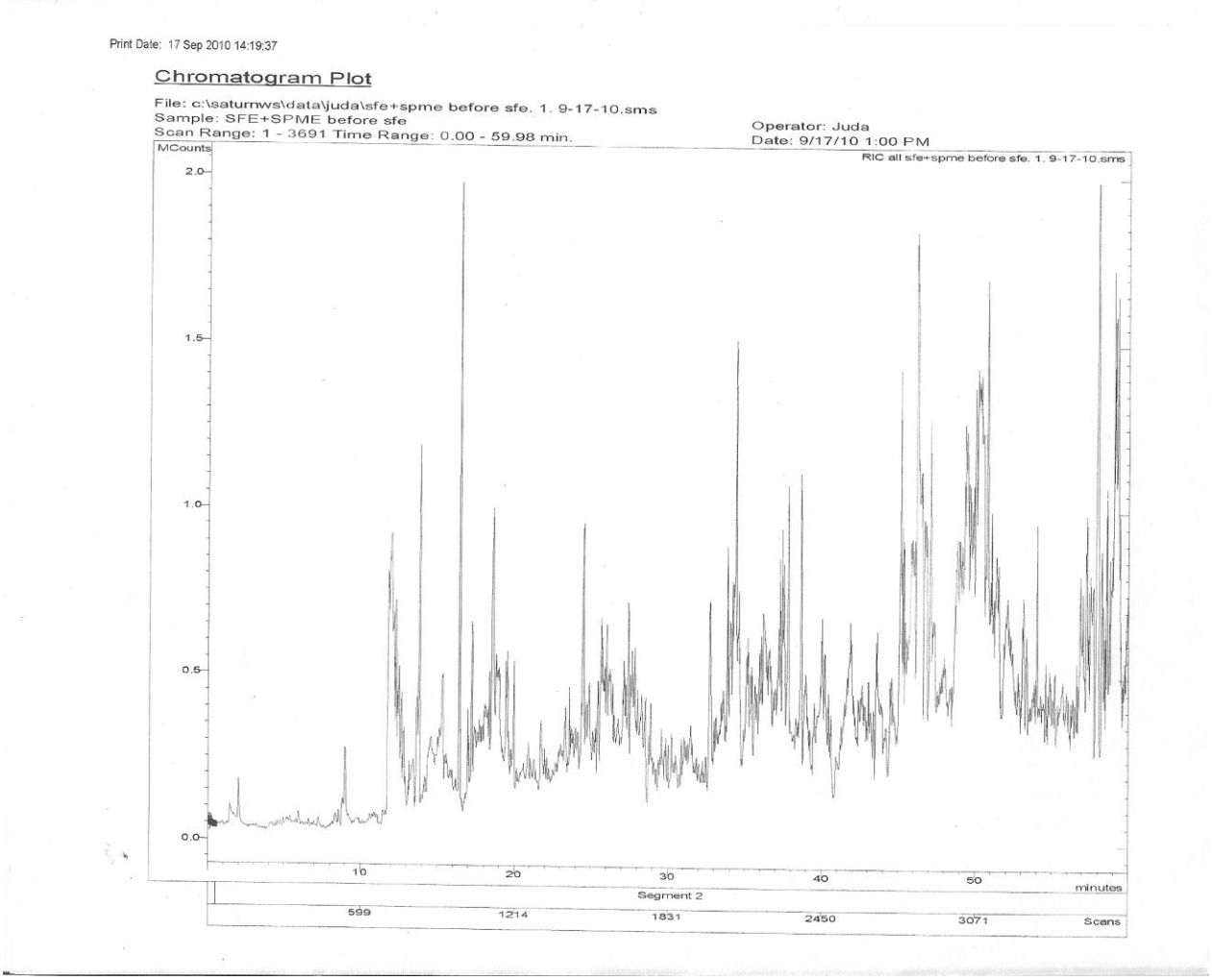
Appendix 2.5 GC separation of an SFE truffle extract collected in isopropanol after 20 minutes of dynamic extraction. GC-FID, column; VF 5ms 30m x 0.25 mm x 0.25 µm



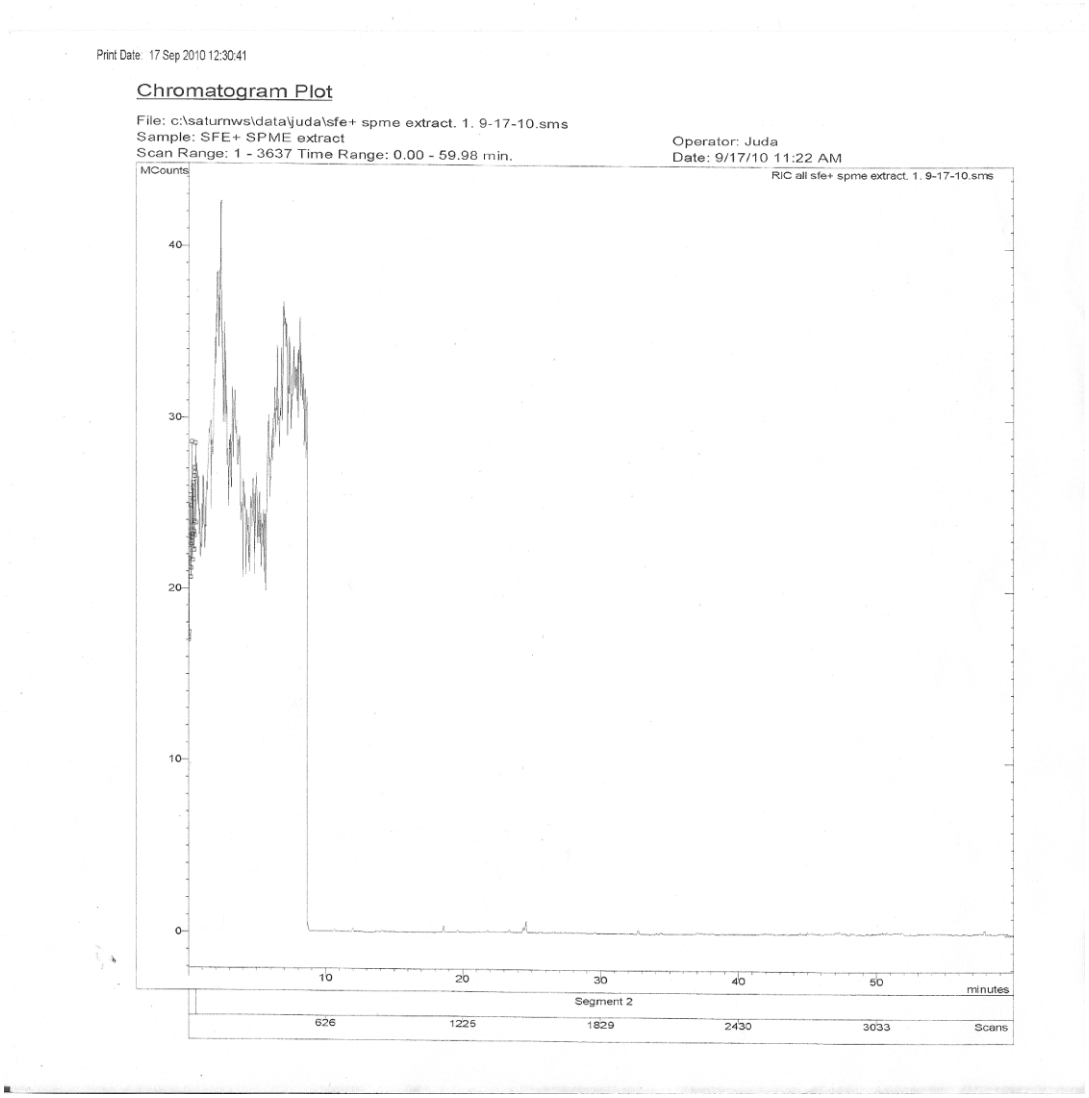
Appendix 2.6 GC separation of blank PDMS 100 µm fiber after conditioning GC-FID, column; VF 5ms 30m x 0.25 mm x 0.25 µm



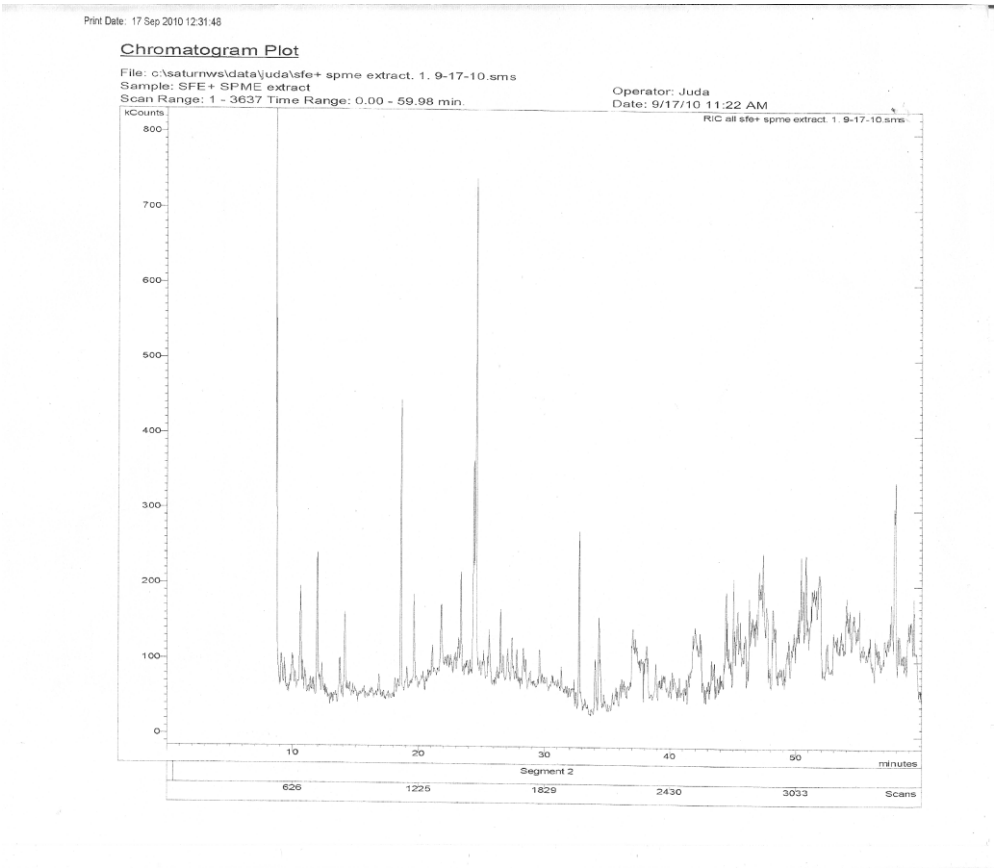
Appendix 2.7 GC-FID separation of PDMS 100 µm fiber truffle extract before SFE, column; VF 5ms 30m x 0.25 mm x 0.25 µm



Appendix 2.8 GC-FID separation of PDMS 100 µm fiber extract after SFE truffle extract collected in isopropanol after 20 minutes of dynamic extraction, column; VF 5ms 30m x 0.25 mm x 0.25 µm

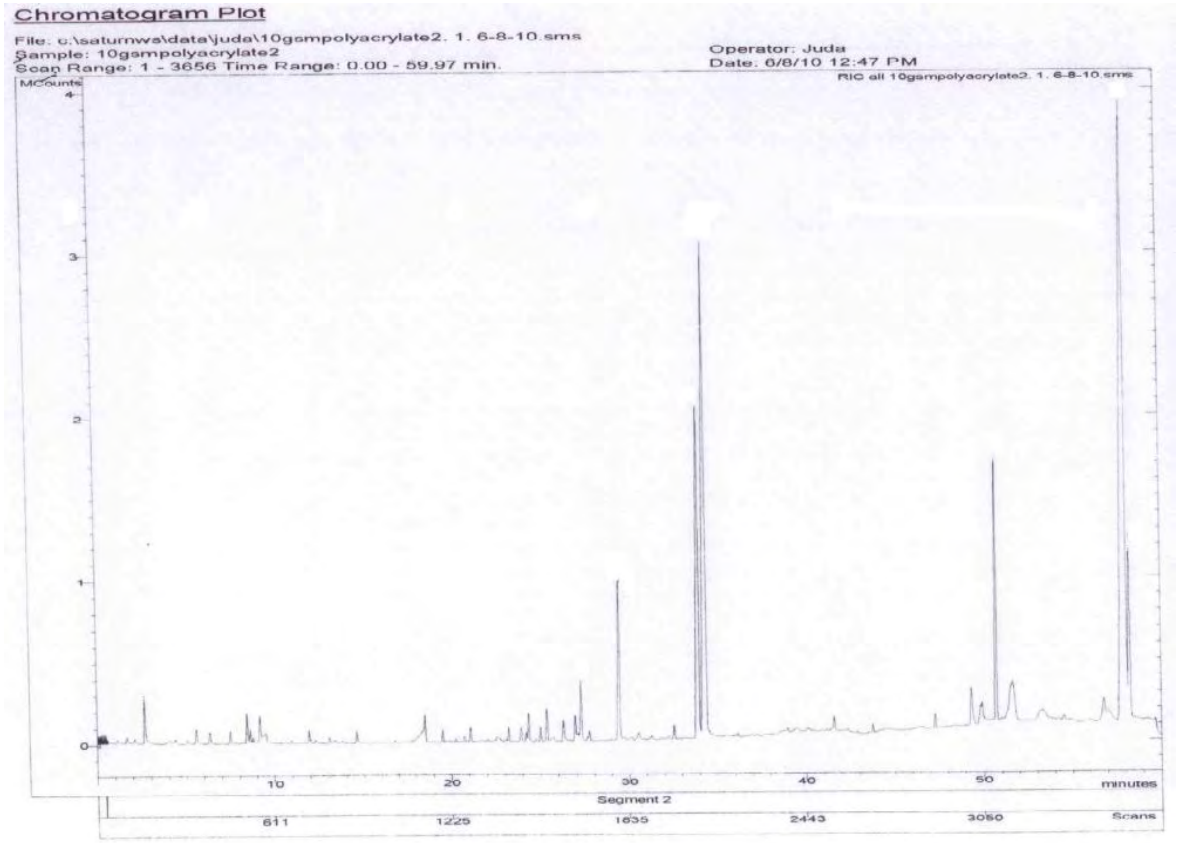


Appendix 2.9 zoomed in GC-FID separation of PDMS 100 µm fiber extract after SFE truffle extract collected in isopropanol after 20 minutes of dynamic extraction, column; VF 5ms 30m x 0.25 mm x 0.25 µm



Appendix 3.1 - 3.3 Shows separated peaks and tables of identified compounds extracted by polyacrylate 85 µm PDMS 7 µm ,and DVB/CAR/PDMS (50/30 µm) SPME fiber

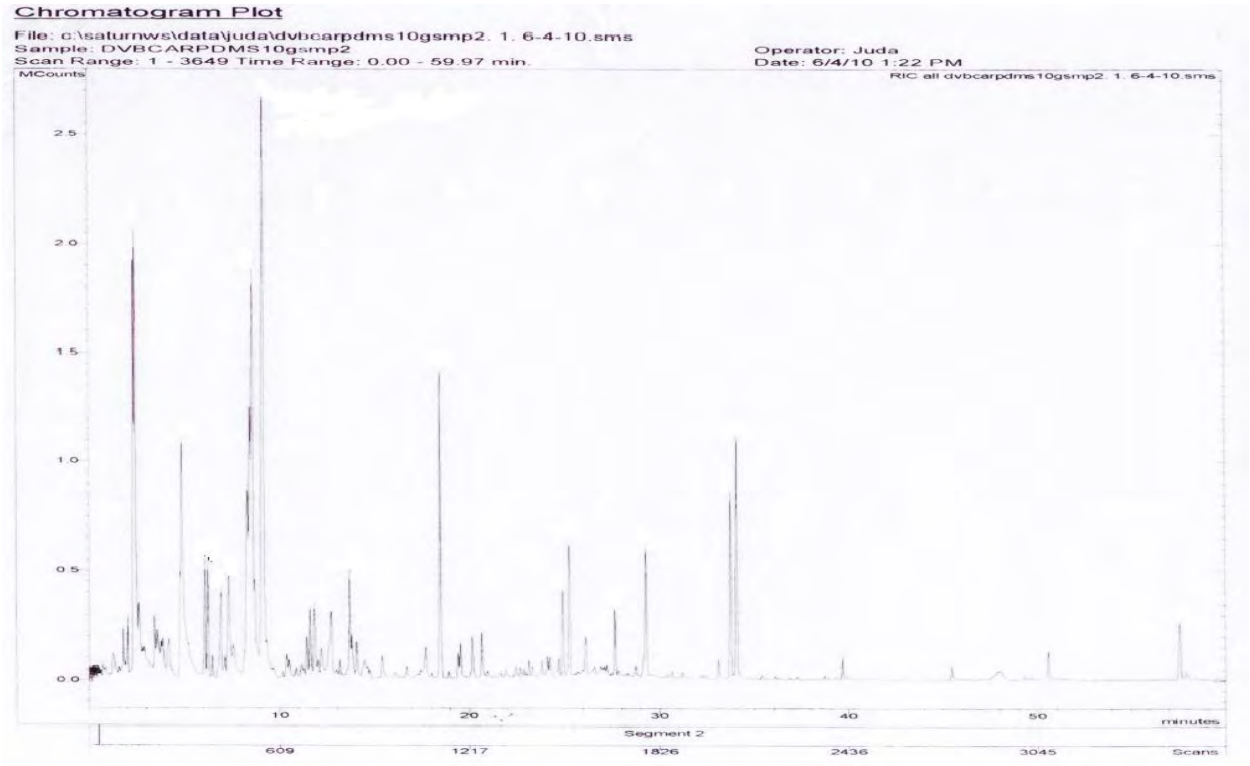
Appendix 3.1 separated peaks and table of identified compounds extracted by Polyacrylate 85 µm SPME fibre.



Volatile compounds identified in Kgalagadi dessert truffles after sampling with Polyacrylate 85 µm SPME fibre.

Peak No	Name	Mol. Weight	Purity	Fit	Retention time	Area
1	decanoic acid	172	485	765	2.682	1110193
2					27.283	1747021
3	Benzoic acid, 2,4-dihydroxy-6-methyl-,methyl ester	182	569	863	29.471	5640432
4	Benzoic acid, 2,4-dihydroxy-3,6-dimethyl-,methyl ester	196	606	929	33.990	16229652
5	Benzoic acid, 2,4-dihydroxy-3,6-dimethyl-,methyl ester	196	577	893	34.394	28089902
6	Hexadecanoic acid, ethyl ester	284	509	958	50.722	7397584
7	8,11-octadecanoic acid, methyl ester	294	462	970	58.083	57292044
8	Ethyl oleate,	310	377	975	58.270	14154469
	Totals					**131661297**

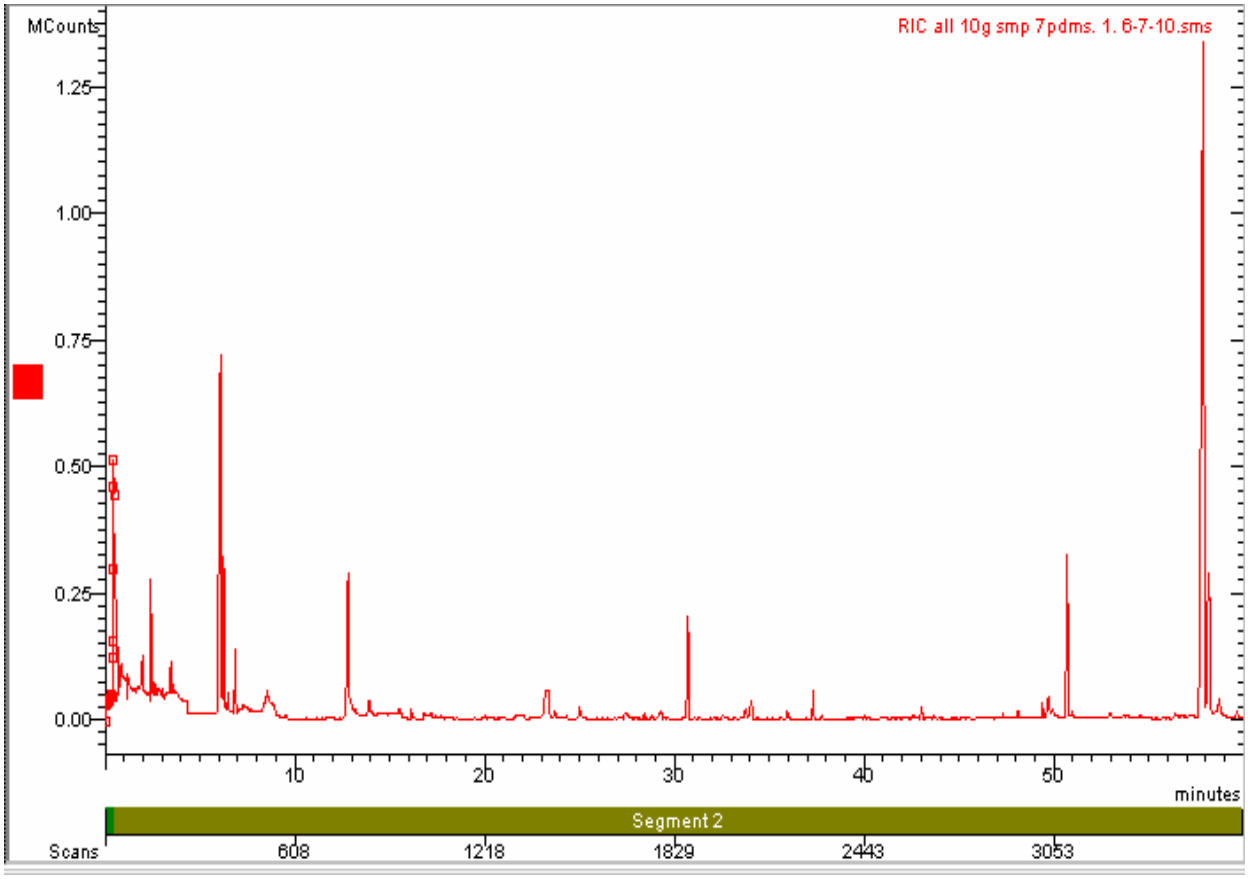
Appendix 3.2 separated peaks and table of identified compounds extracted by DVB/CAR/PDMS (50/30 µm) SPME fibre.



Volatile compounds identified in Kgalagadi dessert truffles after sampling with DVB/CAR/PDMS (50/30 µm) SPME fibre

Peak No	Name	Mol. Weight	Purity	Fit	Retention time	Area
1	2-t-butyl-2,3-dimethyl-3-buten-1-ol	156	220	910	2.443	9950323
2	Styrene	104	883	982	4.872	8985139
3	4-nitro ethylbenzene	151	376	861	6.116	2175453
4	Cyclohexanone	98	726	778	6.952	2422238
5	2-heptenal (E)	112	653	934	7.361	3410814
6	3-heptanone, 6 methyl	128	222	827	8.604	12761965
7	Isoquinoline, 1 butyl-3,4-dihydro	187	597	844	9.175	24274568
8	Ethyl heptanoate	158	620	930	13.666	2148651
9	Octanoic acid ethyl ester	172	360	821	18.489	6651349
10					24.937	1837876
11	5-isopropyl-8methyl-5,6,7,8 tetrahydro-2,4-quinazolinedione	222	440	954	25.294	3068481
12	Ethyl palmitate	284	594	923	27.682	1282333
13					29.302	2820387
14	Bezoic acid, 2,4-dihydroxy-3,6-dimethyl- methyl ester	196	638	885	33.743	4054904
15	Bezoic acid, 2,4-dihydroxy-3,6-dimethyl- methyl ester	196	575	903	34.069	5765060
16	9,12-octadecadienoic acid (22)- 2,3 dihydroxy propyl ester	354	721	957	57.592	1758842
	Totals					**93368383**

Appendix 3.3 separated peaks and table of identified compounds extracted by PDMS 7 µm SPME fibre.



Volatile compounds identified in Kgalagadi dessert truffles using PDMS 7 µm fiber.

Peak No	Name	Mol. Weight	Purity	Fit	Retention time	Area
1	Silane, methylenebis	76	592	867	0.463	4022132
2	[1,1-Biphenyl]-3aminium, 2hydroxy-N,N,N-trimethyl-hydroxide, inner	227	90	885	2.430	500327
3	benzene,1,2-dimethyl-4-nitro	151	350	907	6.066	4635529
4	Proline, 2-methyl-5-oxo-methyl ester	157	399	963	6.823	409459
5	Cyclotrisiloxane,hexamethyl	222	698	924	12.816	2147289
6	Silane, [bicyclo[4,2,0]octa-3,7-diene-7,8-diylbis(oxy)]bis-trimethyl	282	629	814	30.712	1004372
7	hexadecanoic acid, ethyl ester	284	633	944	50.690	1332714
8	8,11-octadecadinoic acid, methyl ester	294	488	984	57.874	11904376
9	Tetradecanoic acid, 12-methyl-(s)	242	258	935	58.161	2229869
	Totals					**28186067**