‘WE DON’T NEED YOUR BLOOD’: Revealing the Opportunities in the Complex and Surprisingly Individualised Volatile Organic Compound Profiles of Human Skin and Saliva

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ABSTRACT

Non-invasive techniques for *in-vivo* sampling of volatile organic compounds (VOCs) from human skin and saliva have been developed and characterised using polydimethyl siloxane (PDMS) as a sorptive material. These samplers have been analysed by thermal desorption (TD) in combination with gas chromatography – mass spectrometry (GC-MS) and applied to global, untargeted metabolic profiling studies of skin and saliva. The utility of an existing PDMS 'Skin Patch' for untargeted metabolic profiling studies has been evaluated and background artefact levels and sample storage stability shown to be key considerations. Successful storage of skin samples for up to 21 days at -80 ºC has been demonstrated.

Evaluation of published methods reveals that existing methods for saliva sampling do not address the physical–chemical attributes of VOCs and evidence is presented for adopting *in situ* sampling of salivary VOCs directly from the oral cavity. *In-vitro* studies indicated that the vapour pressure of analytes was a factor in both the recovery of analytes and the precision of the recovery. The highest recoveries were observed for VOCs with the lowest vapour pressures, for example 5-nonanol (vapour pressure (Pv= 14 Pa) recoveries were approximately 20 times greater than those observed for octane (Pv = 1726 Pa). Similarly, relative standard deviations reduced with vapour pressure, with the % relative standard deviation (RSD) for 5-nonanol responses observed to be 2.7% when compared to 26% for octane. Evaluation of VOCs recovered from *in-vivo* samples (n = 6) indicated that VOC concentrations in saliva may follow lognormal distributions. Comparison of *in-situ* sampling with passive drool saliva collection revealed up to 10^5 fold concentration enhancement with reduced variability compared to drooled samples.

Evaluation of data processing workflows highlighted the challenges in automating data handling for the complex data sets produced; dynamic background compensation (DBC), deconvolution and retention index alignment were required to create a data matrix for multivariate analysis.

The samplers and workflows have been applied in metabolomic profiling studies on stress. Four potential skin VOC markers of psychological stress have been tentatively identified and a principle component analysis (PCA) model demonstrated good predictive capability for stressed and un-stressed individuals.

Potential biomarkers of physiological stress have been tentatively identified in skin and saliva VOC samples collected before, during and after exercise. A suite of candidate markers in skin (n = 8) and saliva (n = 7) has been shown to differentiate between individuals in differing stages of relaxation, physical exertion and recovery. The complementary datasets produced from the different sample types using the workflows developed and described in this thesis has been highlighted and the potential for combining VOC profiles to build a person's 'volatolome' discussed.

LIST OF ABBREVIATIONS

%RSD	% Relative standard deviation
ANOVA	Analysis of Variance
BP	Blood Pressure
COPD	Chronic Obstructive Pulmonary Disorder
Da	Dalton
DC	Direct Current
DNPH	2,4-Dinitrophenylhydrazine
EIC	Extracted Ion Chromatogram
ESI	ElectroSpray Ionisation
FID	Flame Ionisation Detector
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
IMS	Ion Mobility Spectometry
mL	Millilitre
m/z	Mass – to – Charge Ratio
MS	Mass Spectrometry
MVA	Multivariate Analysis
N	Number of Observations
Par	Pareto Scaled
PASAT	Paced Auditory Serial Addition Task
PC	Principle Component
PCA	Principle Component Analysis
PLS-DA	Partial Least Squares – Discriminant Analysis
PDMS	PolyDiMethylSiloxane
PPMV	Parts Per Million by Volume
PPTV	Parts Per Trillion by Volume
PTFE	PolyTetraFluoroEthylene
QC	Quality Control
RF	Radio Frequency
SPME	Solid Phase MicroExtraction
STE	Sorptive Tape Extraction
SVOC	Semi-Volatile Organic Compound

TIC	Total Ion Current
TLC	Thin Layer Chromatography
ToF	Time of Flight
UV	Ultraviolet
VFA	Volatile Fatty Acid
VOC	Volatile Organic Compound
WHO	World Health Organisation

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1 INTRODUCTION: THE HIDDEN CODES IN HUMAN SCENT

1.1. VOLATILE ORGANIC COMPOUNDS AS MARKERS OF HUMAN FUNCTION

1.1.1. The role of olfaction in disease detection

The scents of mammals are complex blends of natural products that can reveal a wealth of individual information [1]. The most evocative and nostalgic of all the human senses, smell can affect a person's mood, memory and whole sense of well-being; a house filled with familiar comforting aromas such as baking bread and log fires can make us feel instantly at home and safe [2] [3]. The sense of smell relies on specialised sensory receptors in the olfactory system perceiving volatile compounds and humans are able to distinguish over 10,000 odour molecules and apply this information in everyday life, often without realising – testing whether food is fresh to eat for example [4].

The application of the sense of smell to medical diagnosis is likely to have originated with the father of modern medicine, Hippocrates, around 400 BC, long before the germ theory of disease had developed. Medical practitioners described how they were able to perceive that diseases in humans changed the odour of bodily excretions [5]. This understanding of the relationship between odours and disease states can be traced through history to ancient Greek apothecaries, who employed urine charts linking taste, smell and colour to known medical conditions, and traditional Chinese medical practitioners who poured sputum onto hot coals and identified a unique scent indicative of tuberculosis [5-7].

The link between olfactory perception and disease diagnosis is not limited to our more primitive ancestors; diagnosis based on smell remained one of the most reliable methods used in bedside medicine for many years. In 1982 Fitzgerald and Tierney, concerned that this skill would be lost in translation to future generations of medical practitioners, felt this area to be of such import that they documented a list of descriptive aromas for particular human diseases and disorders [5]. An excerpt is included in Table 1.1.

On the battle field in World Wars One and Two physicians had no option but to rely on their sense of smell to identify gas gangrene and even today warning signs of liver disease include a fishy smell from patients and the 'fruity' smelling breath caused by diabetic ketoacidosis is employed in emergency rooms for rapid, potentially life saving diagnosis [7].

Table 1.1 Early uses of descriptive aromas in diagnosing diseases and metabolic disorders, adapted from reference 5.

Disease / Disorder	Body Source	Descriptive Aroma
Anaerobic infection	Skin / Sweat	Rotten apples
Cystic fibrosis	Infant stool	Foul
Diphtheria	Sweat	Sweet
Lung abscess	Breath / Sweat / Urine	Foul, putrid
Renal failure	Breath	Stale urine
Rubella	Sweat	Freshly plucked feathers
Typhoid	Skin	Brown bread
Yellow fever	Skin	Butcher's shop

Whilst human olfaction has been employed with some success in this field humans, have long relied on the far superior canine sense of smell; bloodhounds have reportedly been used in tracking humans based on their scent since the sixteenth century! Canine scent plays a fundamental role in their existence and this has been exploited by humans in many ways, the principle among these being tracking, drug detection, search and rescue and forensic applications based on the premise that the individuality of human scent can be distinguished by trained dogs [8]. Dutch researchers have shown the scent from the human palm to be the most individualised and trained police dogs have been shown to match individuals to their clothing by matching the scent from their palms with the pockets on the clothing [9].

The hypothesis that the 'smell of cancer' in humans can be distinguished by dogs was proposed as early as 1989 but has recently caught public imagination with a number of articles appearing in news items, one notable publication boasted the headline 'Paging Dr Dog…' in the Wall Street Journal (10.07.2012). Williams and Pembroke first reported the observation of an untrained dog aiding in identifying a melanoma, reportedly the dog kept 'worrying' a mole on its owners leg and ultimately tried to bite it off, this caused the owner to seek medical advice which resulted in

diagnosis and treatment for the cancer [10]. Several examples of trained dogs differentiating between diseased and healthy controls have been reported with sensitivities from 88-100% and specificities from 91-99% for tissues from ovarian tumours and melanoma, exhaled breath for lung, breast and colorectal cancers, urine for prostate cancer and faeces for colorectal cancer. For more detail on specific studies the reader is referred Buszewski and co-workers review on unconventional methods of cancer detection using odour [8].

There are also a number of anecdotal reports of dogs raising the alarm when their owners become hypoglycaemic [7]. The reason behind a dog's reaction to these disease states is currently unknown, yet it is commonly accepted that something in the volatile emanations from these individuals is detected by canines and elicits a reaction.

While it is evident that the canine sense of smell is in many aspects superior to current analytical instrumentation in detecting and processing this kind of data, there must be metabolic changes associated with the disease states that alter the volatile profile from human sufferers and therefore with suitable instrumentation and data processing an objective measure of these changes should be a realistic research goal.

1.1.2. The advent of biomarker discovery

The invention of techniques for separating complex mixtures, in particular gas chromatography (GC), during the 1950s and their subsequent coupling with mass spectrometry (MS) provided instrumentation to begin to address the challenge of separating and identifying the volatile organic compounds (VOCs) responsible for the changes in odour with disease states [5]. The World Health Organisation classifies VOCs as those compounds with boiling points within the range 50-100ºC to 240-260ºC and semi-volatile organic compounds (SVOC) as those compounds with boiling points within the range 240-260ºC to 380-400ºC these classifications will be adopted for the purposes of this thesis [11]. It is now understood that the changes in odours associated with biological fluids or tissues are due in part to metabolic changes within the body and may be indicative of an individual's state of health, such was the birth of metabolomics and metabonomics [12].

Metabolomics and metabonomics both study the collection of metabolites from a cell or organism, known as the metabolome. The distinction between the two terms is tenuous and numerous definitions can be found in the literature, one such attempt to differentiate between the two is based on the choice of technique stating 'metabolomic studies typically utilise MS whilst the term metabonomics typically describes studies performed with nuclear magnetic resonance (NMR) spectroscopy' [13]. Another review defines metabonomics as aiming to measure the global,

dynamic metabolic response of living systems to biological stimuli or genetic manipulation, and metabolomics as seeking an analytical description of complex biological samples, aiming to characterise and quantify all the small molecules in such a sample [6]. In practice the same analytical and data processing procedures are followed in both and the terminology is generally used interchangeably. In this thesis all studies of this kind will be referred to as metabolomic studies.

VOCs are released by biological systems and can be considered as a kind of terminal metabolite [14] representing the end points of gene expression and cell activity. Thus metabolomics offers a holistic approach to exploring the phenotype of an organism [15].

Early practitioners of metabolomics used paper chromatography to characterise individual metabolic patterns in human biological fluids. This pioneering work was soon followed by thin layer chromatography (TLC) [15]. The complexity of the challenge was soon recognised and the need for enhanced separation, improved sensitivity, and automated analytical and data handling procedures led to extensive research resulting in a number of analytical techniques employed in metabolomic studies. For the volatile signatures associated with the changes in odour with disease state the 'gold standard' technique is GC, preferably coupled with MS.

A number of biological matrices have been employed in metabolomic studies for human biomonitoring. Blood directly reflects the internal environment of the body including nutritional, metabolic and immune status [16] and is less affected by exogenous contaminants than most other biofluids. As a result whole blood, serum and plasma have been employed in biomarker prospecting studies [17] [18]. Obtaining blood samples is not easy, patients find it invasive and often painful, there is an increased risk of infection due to the inevitable puncture wounds and the necessity for stringent storage and pre-treatment procedures makes blood sampling a time consuming and expensive method.

A recent headline in the British press read *'Half of UK population will get cancer in lifetime'* (BBC News 07.06.2013). This highlights the need for effective screening techniques for early diagnosis. Currently screening based on imaging methods, such as ultrasound and mammography, are employed to select patients suspected of having cancer but ultimately the diagnosis can only be made on the basis of tissue biopsy and histopathological examination [8]. Whilst an effective method, the drawbacks associated with diagnosis by tissue biopsy are much the same as with blood sampling and neither of these present an acceptable solution for widespread routine, in-field or in-clinic screening of large populations. Widespread in-clinic screening is essential for successful early diagnosis, and must be the end-goal of research into early cancer, or indeed any

disease, diagnosis. A desirable screening method should be non-invasive, painless, easily accessible and inexpensive but, above all, it should be sensitive, reliable and highly selective for markers of the disease to facilitate early stage diagnosis of the disease state [8].

Identified as early as 400 BC the volatile emanations, or smell, from human beings may contain useful information about an individual's state of health. Once relationships between VOC profiles and biological pathways are understood VOC profiles will transcribe complicated bio-information [14] and may provide a route to the next generation of non-invasive screening techniques. To this end significant research effort has been invested in identifying and developing non-invasive sampling techniques for the measurement of human function and applying them to metabolomic studies.

1.1.3. Non-invasive sample collection for metabolomics

The natural starting point for a non-invasive sample is a matrix that is regularly excreted from the body; urine and faeces for example. Biomarkers for cholera [19], inflammatory bowel diseases [20] and colorectal cancer [21] have been tentatively identified in faecal samples, however these samples are more routinely used in monitoring exposure to pesticides or biomonitoring of metals than in diagnostic screening [22].

Urine samples have found application in many areas of bioanalysis including, but not limited to, pharmacokinetic studies of administered pharmaceuticals, detecting drugs of abuse, doping in sports, and measuring exposure to xenobiotic substances [18] [23]. Due to the ease of collection and relative volume of samples urine has also been studied extensively in metabolomics and biomarkers have been proposed for chronic obstructive pulmonary disease (COPD) [24] and a number of cancers including prostate, bladder, breast, ovarian, cervical and renal [25] [26]. Direct analysis of urine is limited by high concentrations of urinary salts so a clean-up stage and consideration of pH control is required prior to analysis [22]. The variations in excreted volumes, and hence concentrations, also necessitate normalisation as part of the analytical work flow; most commonly achieved by the creatinine concentration adjustment method [27]. As with blood samples storage conditions are paramount in preserving the integrity of urine and faecal samples prior to analysis, typically low temperatures (-80 °C) are required.

For many people faeces and urine are bound up with taboo and providing such samples can be discomfiting and embarrassing for patients, and this may result in problems with patient compliance. These approaches cannot necessarily be considered as being completely non-invasive, the term 'minimally invasive' may perhaps be more appropriate.

Sputum has been employed with some limited success in metabolomics and biomarkers for asthma [28], COPD [29], lung cancer [30] and tuberculosis [7] have been described. Whilst sputum may be considered non-invasive, because there is no requirement for tissue damage to collect the sample, sputum induction requires salbutamol ingestion followed by inhalation of hypertonic saline solution [28]. This combination causes the patient to cough violently so that sputum is ejected from the lower airways into the mouth for collection. At best this procedure can be described as semi-invasive and, as with blood sampling and tissue biopsy, is relatively expensive and requires trained medical personnel to perform the sampling.

Skin cancer is currently both the most common and the fastest growing cancer in the United Kingdom [31]. If melanoma is detected early enough the 5 year survival rate is around 98.2% [32]. Unfortunately the majority of cases are not detected in this time window and some of this may be attributed to patients' reluctance to bother their general practitioner (GP) with 'just a mole', or to the fear of unnecessary biopsy or hospitalisation. The idea that VOC profiles may contain signatures from melanoma is not a new one and headspace sampling of excised melanoma, naevi and healthy skin has shown volatile markers that differentiate between them [12] [32] and provides a proof of principle that diagnosis of malignant melanoma may be possible *in-vivo.* Other local skin conditions have also been explored in conjunction with skin profiles including atopic dermatitis [33], chronic wounds [34] and allergens from skin creams [35]. Skin sampling has even been applied to studies of systemic diseases; a marked difference in metabolic activity was observed in skin fibroblasts from Alzheimer's sufferers and preliminary studies have shown indications that dermal markers may reflect the presence of breast cancer [33]. One study of emanations from human skin has shown acetone levels to correlate with circulating blood glucose which may provide the basis for a non-invasive monitoring technique for Type 1 diabetes sufferers [36].

Studies on volatile emanations from the skin surface have not been limited to diseases and disorders, numerous studies characterising body odour [37] [38], mosquito attractants [39] [40], gender differences [41], age-specific odours [42], fragrance release [43] and even indicators of seasonal changes [44] have all been studied non-invasively by various collection and analytical techniques. The skin sampling techniques that have been described are many and varied and will be covered in more detail later in this chapter, at this point it is sufficient to comment that these studies all highlight the low concentration of some potentially significant skin emanations so non-invasive sampling from human skin requires an enrichment step to harness the full potential indicated by these preliminary studies [45]. Volatile profiles from human skin have been shown to be highly complex and individualised and early indicators point towards useful, diagnostic information on

human function present in such profiles that may prove key in the drive towards non-invasive sampling for early diagnosis based on VOCs.

Human saliva is an easy to collect, low-cost matrix useful for screening large populations without the need for trained clinical staff. The pace of research into saliva as a diagnostic medium has accelerated in recent years as the non-invasive nature makes it preferable for the patient compared to blood, biopsy, urine or faeces [46] and quantitative studies have indicated meaningful correlations between blood and saliva concentrations for non-protein bound analytes [47-49]. Collection is straightforward and comfortable and saliva sampling and analysis has been used to: monitor exposure to hazardous chemicals [50] [51], identify drugs of abuse [52] [53] and study dietary factors [54-56]. In metabolomic studies biomarkers for a number of systemic conditions have been proposed including renal disease [57], gout [58], cystic fibrosis, diabetes mellitus, kidney dysfunction and alcoholic liver sclerosis [59].

Whilst skin and saliva sampling have demonstrated promise as potential non- invasive sources of biologically relevant information the greatest focus in this area in recent years has been on VOCs in exhaled breath [60].

Exhaled air is a mixture of nitrogen, oxygen, carbon dioxide, inert gases, water vapour and trace level VOCs [61] and the non-invasive, safe and straightforward nature of the sampling process makes breath analysis an attractive tool for clinical screening and metabolomic studies [62]. Numerous techniques for capturing the VOC profile in exhaled breath have developed in recent years but the first quantitative measurement of alveolar breath was developed as early as the 1930s – the Haldane-Priestly sample tube, and variants, has been used in a number of studies on metabolic disorders [63]. Two main approaches have been used for sampling exhaled breath, real-time analysis which requires a participant to expire directly into the analytical instrument gives the advantage of real-time breath by breath measurements. The concentrations of potentially significant VOCs in breath can range from ppmv to as low as pptv [63] and so the majority of modern breath samplers employ a pre-concentration step either by collecting breath into a canister or sampling bag followed by cryofocussing, or by passing breath through an adsorbent trap [60].

Volatile markers for a number of lung diseases have been proposed in exhaled breath samples; elevated levels of hydrocarbons have been found in patients with asthma, COPD, obstructive sleep apnea, cystic fibrosis and pneumonia [60] while a number of methylated hydrocarbons have been proposed as markers of lung, breast, colorectal and prostate cancer [64] [65]. Metabolic disorders have also been explored in relation to breath VOC profiles and levels of breath acetone have been

shown to correlate with blood glucose levels in diabetic patients [60]. Other, non-clinical, applications of breath samples in metabolomic studies have included the variation in VOC profiles with diet, environment, exercise and even induced psychological stress [66-68] [62].

Whilst breath sampling appears to offer many advantages in the drive for non-invasive large-scale screening techniques, obtaining a reproducible and representative breath sample is not as simple as it may initially seem; exhaled air comprises a mixture of dead space air – approximately 150 mL from the upper airway where there is no direct gaseous exchange with circulating blood, and alveolar, or end-tidal air. Dead space air offers little information about the metabolites circulating in blood and is often heavily contaminated with exogenous compounds recently inspired, whereas alveolar air, originating in the lower respiratory pathways, is in contact with circulating blood and rich in endogenous VOCs. Three modes of breath sampling then present themselves; whole breath sampling – a mixture of dead space and alveolar air, dead space air only or alveolar air only. For diagnostic screening or metabolomic studies the obvious choice is alveolar only. Unfortunately this complicates the sample collection strategy, end-tidal breath is often diluted by dead-space air and sophisticated techniques that sample only alveolar air are required. A number of samplers have been designed to this end the more sophisticated of which employ a CO_2 controlled valve [69] or pressure sensor [63] to mark the transition between dead space and alveolar breath. While these techniques facilitate high quality, reproducible breath samples the additional complexity and training required to obtain the samples reduces the applicability of the sampling to large scale routine screening. For remote sampling a number of single-breath sampling methods have been employed, however it is difficult to achieve sufficient sensitivity and reproducibility from a single, or several, breaths.

High levels of water vapour in breath samples can cause complications with sampling, polar species dissolve in condensation and so may not reach the sample collection vessel and water can block adsorption sites in sampling media. Table 1.2 outlines the principle biological matrices for human health and disease monitoring and their main advantages and disadvantages as a sample for large scale clinical screening.

Table 1.2 Overview of sample matrices for disease diagnosis.

	Blood	Tissue (Biopsy)	Faeces	Urine	Sputum	Skin / Sweat	Saliva	Breath
Invasive	Yes	Yes	Minimally	Minimally	Semi	None	None	None
Privacy concerns?	None	None	Yes	Yes	Some	None	None	None
Minimum sample size	>50mL	N/A	>50mL	>50mL	mL	N/A / (µL)	mL	>50mL
Pretreatment?	Yes	Yes	Yes	Yes	Yes	Some methods	Some methods	Some methods
Self administrable?	No	No	Yes	Yes	No	Yes	Yes	Some
Easily Fieldable?	No	No	No	Yes	No	Yes	Yes	Yes
Storage requirements	-80°C	-80°C	-80°C	-80°C	-80°C	Variable	Variable	Variable
Used in routine methods?	Yes	Yes	No	Yes	Yes	No	Yes DNA only	Yes
Suitable for vulnerable participants?	No	No	No	Yes	No	Yes	Yes	Yes

The potential of biological VOC profiles in moving clinical screening towards simple, safe and sensitive non-invasive sampling techniques has reached a proof-of-principle, demonstrated by the studies detailed in this section. The challenges in advancing this field of research principally lie in the benefits of standardisation. A recent drive for more robust studies in breath metabolomics lead to the publication of a workflow for the metabolomic / metabonomic investigation of exhaled breath [70] providing a basic dynamic framework upon which a set of standardised guidelines for breath VOC analysis in metabolomic studies can be built and amended / improved upon in an iterative process.

The studies in this thesis focus on skin and saliva as non-invasive sample matrices that potentially offer complementary information to exhaled breath samples and evaluate the potential for standardised sampling and analysis workflows across a number of biological compartments.

1.2. RESEARCH AIMS

This research is based on the following premises:

- There are discernible differences in the biological volatile organic compound profiles emanating from individuals in different metabolic states.
- Complementary and comparable information can be obtained from volatile profiles associated with different biological compartments; therefore less invasive sampling techniques may be substituted for the established puncture wound based sampling for clinical screening.
- The workflow for collecting, analysing and processing data from volatile biomarker discovery studies for non-invasive VOC samples can be applied to different sample types resulting in complementary data sets.

The research aims of this project are to explore non-invasive sampling and analysis of biological VOCs from human skin and saliva, evaluate the steps in this multi-disciplinary work flow including ethical clearance, sampler design and optimisation, pilot studies and data handling and evaluate each process against the current forerunner in non-invasive measurement: exhaled breath analysis.

This introductory chapter will explain the rationale behind the selection of skin and saliva samples by introducing the transport membranes and discussing the biological and physical chemical aspects that create the associated VOC profiles and review the current status of sampling methodologies and metabolomic studies in this field.

1.3. THE ANALYSIS OF HUMAN SKIN

The skin is the largest organ of the human body comprising around 10% of the body mass and covering approximately 1.7 m^2 of an average adult [71]. The skin can be viewed as a highly efficient, self repairing barrier which serves to act as a permeation obstacle preventing ingress of exogenous molecules and regulating excretion from the surfaces of the body. This oft-overlooked, uniquely engineered organ regulates the loss of heat and body water and performs vital functions both as a protector and in homeostasis, [10] but is generally considered rather simplistically as a barrier to 'keep the insides in and the outside out'. Continually regenerating and metabolically active the skin is functionally the most versatile organ of the human body and can in fact be viewed at varying levels of complexity and facilitates selective absorption and excretion of nutrients, waste products and even pharmaceuticals [72]; essential yet unobtrusive, the human skin is the first line of defence and the end of numerous metabolic pathways performing many more functions than a humble blockade against the outside world.

1.3.1. Human skin structure

The skin consists of four main, physically and functionally distinct, layers which connect to the surface by a number of bridging structures an overview of the principal structures may be seen in Figure 1.1 [73]. The innermost of these layers, the hypodermis, is composed mainly of fat microlobules and fibrous collagen and is also known as the subcutaneous fat layer. A relatively thick layer, in the order of millimetres, the hypodermis is found over the majority of the human body and connects the outer skin layers to muscle and bone whilst allowing movement and offering mechanical protection and insulation. Principle blood vessels and nerves pass through the hypodermis to the outer layers of the skin [71].

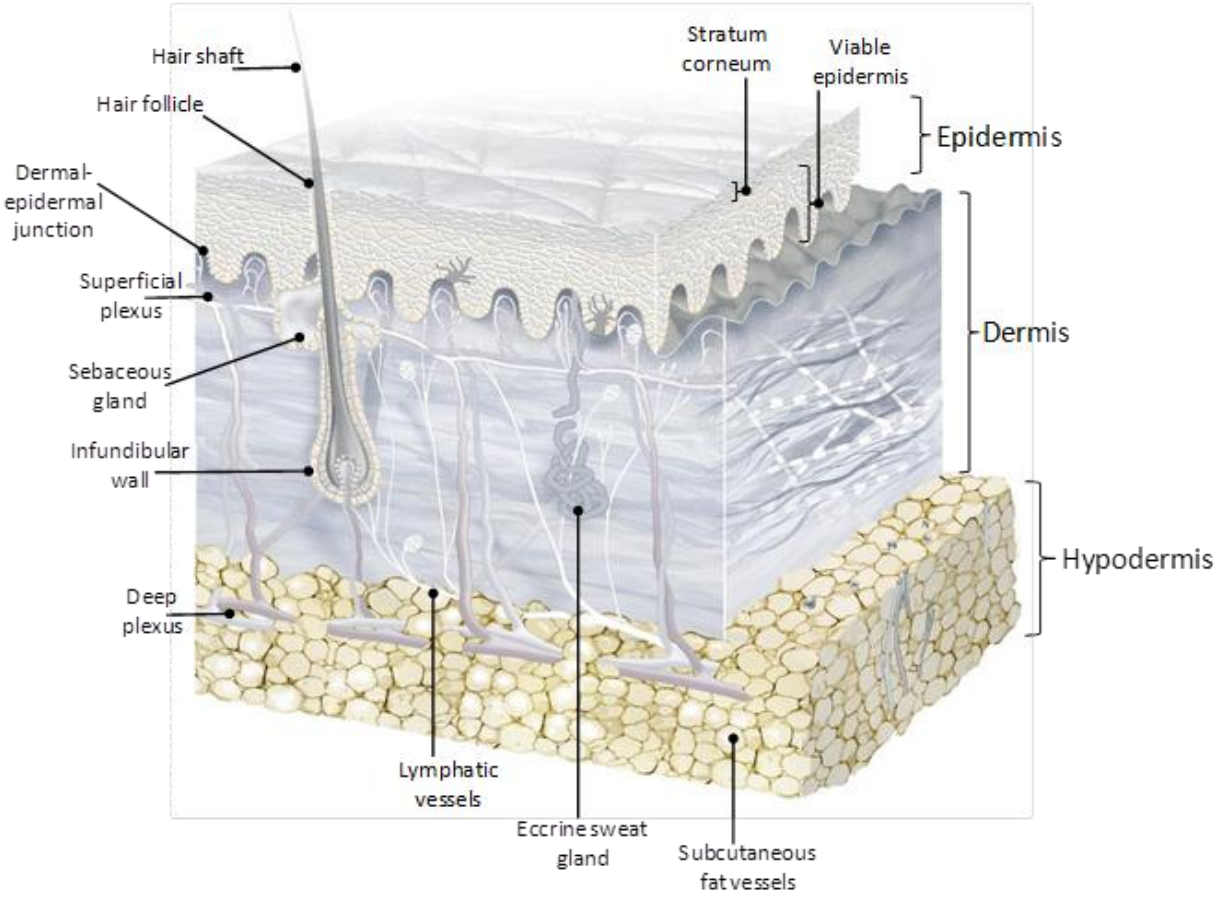


Figure 1.1 **A cross section of human skin showing the principle layers, appendages, blood and lymph vessels, adapted from reference 74.**

Adjacent to the hypodermis lies the dermis which consists of a network of connective tissue and collagen fibrils providing support and elasticity to the skin, two principle layers exist within the dermis; the lower reticular dermis comprising thick collagen fibrils and coarse elastic fibres, and the upper papillary dermis, a mixture of thin collagen bundles, elastin fibres and ground substance [71]. The dermis also houses blood vessels and sections of the lymphatic and nervous system. Skin blood vessels are most concentrated in the papillary dermis as the superficial plexus; a dense collection of arterioles and venules that run along the dermal-epidermal junction. Another, less dense, deep plexus of arteries and veins run roughly parallel deeper in the reticular dermis with a network of connecting blood vessels between the two [74]. Lymphatic system vessels within the skin lie roughly parallel to the blood vessels.

Several classes of surface appendage originate in the dermis, providing bridging links from inner skin layers to the surface:

Hair follicles cover on average 0.1 % of a human body [73] and are distributed evenly over the surface with notable absences on load bearing areas such as the palms of the hands and soles of the feet as well as on the lips and genitals [71]. The conical entrance from the skin surface, known as the infundibulum, extends up to 500 μm into the skin, deeper than the dermal-epidermal junction. A hair follicle joins a sebaceous duct which in turn connects to a sebaceous

gland, sebaceous glands can also be found without associated hair follicles, most commonly on the face. Sebum, a lipophillic skin lubricant, is secreted by the sebaceous glands and fills the infundibulum to provide the skin with protection against bacteria, over wetting and heat loss [75]. The precise composition of sebum varies between individuals and body site and comprises a mixture of triglycerides, fatty acids, wax esters, cholesterol and squalene. The hair follicle is heavily vascularised, the bulb at the base is surrounded by interconnected blood vessels running parallel to the length of the follicle that connect with the vessels surrounding the sebaceous gland [75].

Eccrine glands are a class of sweat gland present from birth and found over the majority of the human body at an average density of 100-200 glands per square centimetre [71], they are particularly numerous on the palms of the hands and soles of the feet with a maximum density of 700 cm^{-2}, absent on the lips and at a minimum on the back with a density of around 64 cm^{-2} [76]. Eccrine sweat is composed of 99% water with the remaining 1% consisting of electrolytes, lactic acid, urea, ammonia, amino acids and a number of protein species [71]. The glands are based in the lower regions of the dermis and connect to the surface by a duct, approximately 100 μm in diameter, which coils around the gland before rising through the outer layers to the skin surface. The main functions of these glands are thermal regulation, improved grip and to sensitise the skin. Secretions are released from the cell as a liquid in response to heat or emotional stress and the composition can vary depending on hydration, state of health and region of the body. Salts and electrolytes can be re-absorbed through the walls of the duct to maintain homeostasis [71].

Apocrine glands are another type of sweat gland connecting the lower skin layers to the surface, apocrine ducts open into the infundibulum in more limited areas of the body such as axillae, nipples and mons pubis [75]. The precise function of these glands is unknown, they have no role in thermal regulation, and they are thought to be relics of odour producing glands in mammals for territory marking and sexual attraction. A milky, viscous fluid is excreted from apocrine glands which is rich in proteins, carbohydrates and ammonia, although sterile apocrine sweat is odourless, bacterial degradation on the skin surface results in the odour of sweat. *Apoeccrine glands* appear during adolescence and have common features with both eccrine and apocrine glands [71].

The outermost layer of skin is called the epidermis and this consists of both viable and non-viable cell layers which, for the purposes of this chapter, will be addressed separately. Epidermal cells undergo continual migration from the dermal-epidermal junction to the uppermost skin layer, the stratum corneum which consists of dead, annucleated, keratinised cells [77]. Between the dermal-epidermal junction and the stratum corneum lies the viable

epidermis, an avascular environment across which desquamation occurs; enzymes degrade viable cell components and nuclei disintegrate before the cells become part of the stratum corneum approximately 14 days after beginning the migration [75].

The final stages of desquamation see the epidermal keratinocytes formed into flat, closely packed corneocytes embedded in a highly organised lipid bilayer matrix; forming the stratum corneum. The lipid composition and organisation in the skin differs to other biological membranes; phospholipids are largely absent and instead eight uniquely structured ceramides together with fatty acids, cholesterol and cholesterol sulphate provide the ampiphillic properties for bilayer formation [71]. The stratum corneum acts as the main physiochemical barrier against uncontrolled permeation both into and out of the skin and is often represented as a 'bricks and mortar' like structure [78].

1.3.2. Skin as a transport membrane

Human skin is more than a barrier; compounds can selectively cross the skin to be excreted in eccrine and apocrine sweat, in sebum and by perfusion from underlying blood vessels giving a rich and highly individualised mixture of metabolites on the skin surface. The transport properties of the skin membrane are exploited in transdermal drug delivery systems such as nicotine patches and hormone treatments, this delivery route offers some unique advantages in that it is non-invasive and self administrable, improving patient compliance, and avoids first pass metabolism by the liver [73].

The skin as a transport membrane can be viewed at varying levels of complexity, the simplest as a single physical barrier, more accurately when the different properties of skin layers are considered; a collection of barriers with differing transport properties arranged in series [71]. This was recognised as early as 1971 by Scheuplein who commented that percutaneous absorption requires absorption into the stratum corneum, and subsequent diffusion through it, the viable epidermis, the papillary dermis and into the microcirculation. The reverse is true for elimination through the skin. The layers of tissue in the viable epidermis, the dermis and the capillary walls are relatively permeable and for the great majority of substances percutaneous absorption or elimination is rate-limited by the stratum corneum [79].

More accurately still when surface appendages, such as hair follicles and sweat glands, are taken into account the skin can be considered as a collection of barriers with varied physiochemical properties both in series and in parallel. Molecules up to a few hundred Daltons in size can cross the skin and their permeation is a multifactoral phenomenon dependant on many physical, chemical and biological interactions [78].

1.3.2.1. Percutaneous permeation

The principle barrier to permeation through the skin lies in the 10-20 µm thick non-viable cell layer; the stratum corneum, transport across which occurs by passive diffusion [78] [80]. The widely accepted 'bricks and mortar' structure of the stratum corneum, represented in Figure 1.2, offers two pathways for diffusion; the transcellular route requires repeated partitioning of a solute molecule into both lipophillic and hydrophilic compartments and studies have indicated that this is the minor route of epidermal permeation [73] [79] [80].

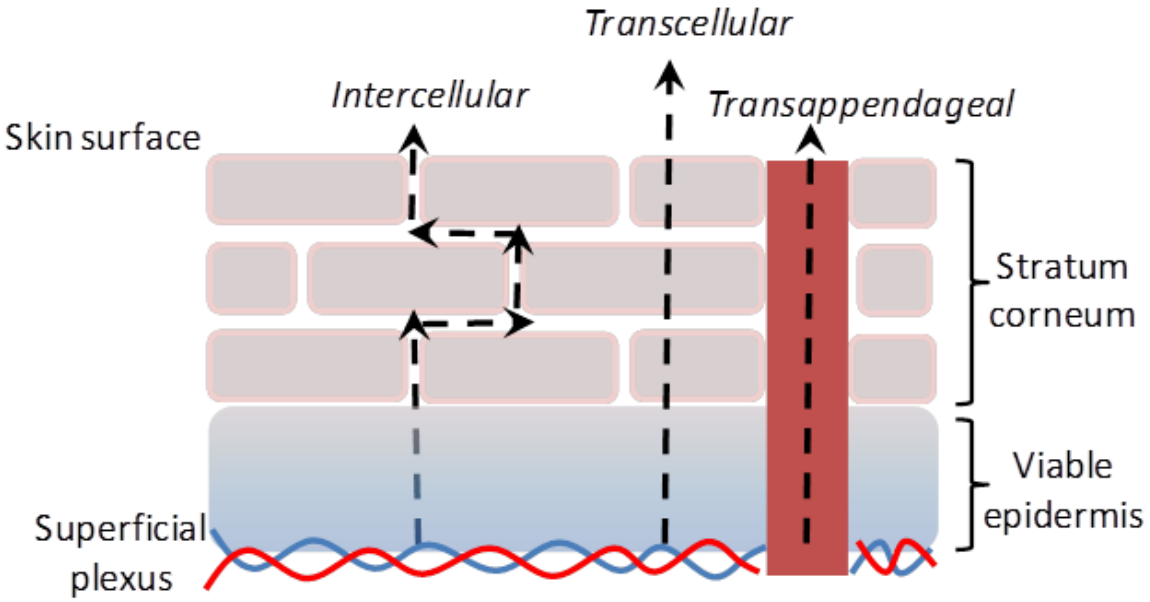


Figure 1.2 A bricks and mortar representation of the outermost skin layer, the stratum corneum. The major routes of transport from circulating blood to the skin surface are shown. Adapted from reference 73.

The second, more widely accepted, diffusion pathway is the tortuous intercellular path that winds around the cellular 'bricks' through the lipid bilayer 'mortar'. Hydrophilic compounds, soluble in the polar head, groups have been observed to have a near constant permeation through the aqueous regions whereas passage of lipophillic compounds transported by diffusion through the non-polar tail groups vary considerably with lipophilicity [73]. The transport of lipophilic compounds through the 'tail' section of the lipid bilayer is highly constrained by structural and solubility properties and can be approximated by the octanol/water partition coefficients, *logP*. Log P values between 1 and 3 are considered ideal to facilitate penetration through the stratum corneum [73]. The third route, transappendageal transport, will be discussed in the next section.

Molecules in the gas phase undergo Brownian motion which induces an overall diffusional flux from areas of high to areas of low concentration in a homogenous medium [80] [81]. For a simple inert membrane the steady state flux, J_{ss} (moles cm^{-2} s^{-1}) is defined by the amount of

substance, Q_{ss} (moles), passing across a surface of area A (cm^2) in a given time period *t* (s) and is related to the concentration difference between the two membrane surfaces, $\Delta[i]$ (moles cm^{-3}), the diffusion coefficient of the solute in the membrane, *D* ($cm^2\ s^{-1}$), and the diffusion path length or membrane thickness x (cm) [81]:

$$J_{ss} = \frac{Q_{SS}}{At} = \frac{D\Delta[i]}{x}$$ **Equation 1. 1**

Skin is not inert and the thermodynamics of adsorption/absorption vary with the type of compound and its concentration. Adsorption / absorption kinetics are determined in part by the solution/membrane absorption isotherm [81]. For dilute solutions, such as those likely to be found in circulating blood or the lymphatic system, these isotherms are linear and obey Henry's law in which absorption is proportional to concentration with the constant of proportionality the partition coefficient between the two phases (***P***):

$$P = \frac{[i]_S}{[i]_C}$$ **Equation 1. 2**

$[i]_S$, Concentration absorbed in skin (moles cm^{-3}), $[i]_C$,Circulating concentration in blood/lymph vessels (moles cm^{-3}), P, Partition coefficient.

This allows the correlation between concentrations across the membrane to be stated in terms of the solute-membrane partition coefficient, *P*. In skin this partition coefficient is generally approximated based on a molecules octanol/water partition coefficient; usually expressed as log P [79].

Permeation across the skin membrane occurs via a solution-diffusion mechanism governed by both Fick's diffusion and Henry's solubility laws [79] [82] and the rate of permeation, or flux, at a steady state, $J_{ss,}$ is related to a solutes partition and diffusion coefficients in skin, the circulating concentration and the skin thickness:

$$J_{ss} = \frac{DP[i]_C}{x}$$ **Equation 1. 3**

The coefficient of permeability, k_p ($cm\ s^{-1}$) is:

$$k_p = \frac{DP}{x}$$ **Equation 1. 4**

For a molecule with a circulating concentration sufficiently low to obey Henry's law the rate of change in concentration at the surface of the skin is proportional to the circulating

concentration and inversely proportional to the skin thickness with the constants of proportionality being the diffusion coefficient of that molecule in the lipid bilayers of the stratum corneum and the partitioning coefficient between the blood/lymph vessels in which the molecule is carried to the skin, and the intracellular lipid bilayers of the stratum corneum. This model assumes a constant circulating concentration with respect to the time taken for permeation to reach a steady state. During skin sampling this process will be driven by a continual depletion at the skin surface due to partitioning into the sampler. It is more likely that concentrations measured by sampling the skin surface will give rise to time-weighted average concentrations with respect to the circulating concentration.

While diffusion through the stratum corneum usually acts as the rate limiting step in excretion via the skin it is also worth mentioning the contribution from the deeper layers [73]; the viable epidermis consists of ~40% protein, 40% water with the remainder made of a lipids. Diffusion through the viable epidermis can be considered as diffusion through an aqueous medium interspersed with regions of higher lipophilcity, the barrier effects of the viable epidermis in transport across skin can be incorporated into skin permeation models by treating it as an homogenous aqueous layer immediately below the stratum corneum so the total epidermal permeability depends on the permeability of the stratum corneum ($k_{p.SC}$) and the viable epidermis ($k_{p.VE}$) and can be represented by the epidermal permeability coefficient, $k_{p.\mathrm{Epi}}$ [75]

$$\frac{1}{k_{p.Epi}} = \frac{1}{k_{p.SC}} + \frac{1}{k_{p.VE}}$$

Equation 1. 5

Similar to the viable epidermis the dermis can be viewed as gelled water however, there are important differences. While the viable epidermis is avascular the dermis is rich in blood and lymph vessels which contribute significantly to the transport and distribution of molecules in the skin. Vasoconstriction and vasodilatation will significantly alter the clearance and absorption rates of compounds through the skin. The treatment of the stratum corneum and viable epidermis as barriers in series can be extended to include the dermis as a third barrier [73].

Highly hydrophilic molecules with low log $P_{o/w}$ values, such as volatile fatty acids and alcohols, will exhibit low rates of permeation across the stratum corneum. Polar, hydrophilic compounds are commonly reported on the skin surface however and other transport routes may contribute to their presence [83].

1.3.2.2. Transappendageal transport

The models above present skin as a series of homogenous layers between the circulatory system and the skin surface, this is a simplification for a number of structures create bridges from the deeper skin layers to the surface and as such bypass some of the diffusive processes described above [73]. As shown in Figure 1.2 transport across the skin can be considered as a collection of barriers both in series as described in Section 1.3.2.1 and when transappendageal routes are considered, in parallel.

The hair follicles, eccrine, and apocrine glands act as transport links to the skin surface and the composition of their secretions contribute to the chemical cocktail on the skin surface and influence the overall permeability [75-80].

Apocrine sweat glands open into hair follicle infandibular in more limited areas of the body. Secretion takes place as apical budding of cells of the duct and is stimulated in response to emotional stimuli such as anxiety, pain or sexual arousal. Apocrine sweat is a milky, viscous fluid containing proteins, lipids and steroids. Recent studies into the odour of sweat have found a set of apocrine secretion binding proteins that act as carrier proteins for a number of volatile odorous molecules [76]. Volatile fatty acids and sulphurous compounds, accredited as the primary causes of human body malodour, are carried to the skin surface in apocrine sweat as amino acid conjugates where they are subsequently released by bacterial enzymes [84].

Apoeccrine glands are not as well understood as other sweat glands although they share characteristics of both eccrine and apocrine glands. Their function is as yet unknown but they are not thought to play a role in thermal regulation. They secrete a watery substance the composition of which strongly resembles eccrine sweat but has yet to be fully characterised due to the difficulty in differentiating between them and eccrine glands during sweating [84].

Sebaceous glands also open into, although are not limited to, hair follicles and as such the composition of apocrine sweat may in fact be contaminated with sebaceous gland secretions. The true function of these glands has yet to be determined. Antioxidant and antibacterial effects are chief among the theories for sebaceous gland function as well as the carriage of pheromones to the skin surface [85]. These glands secrete sebum, a skin lubricant rich in squalene, wax esters, triglycerides and phospholipids. The sebaceous gland, in common with apocrine glands, is a holocrine gland which means that its secretions consist of cells from the gland itself and it secretes a number of compounds in common with those originating from the epidermis, cholesterol for example, but also a number of compounds unique to sebum on the skin surface including squalene and wax esters [85].

These structures complete the set of transport routes of VOCs from the circulatory and lymphatic system to the skin surface. Re-absorption and biotransformations may occur

throughout the whole structure and the rate of secretion is dependent on a number of physiological and psychological factors that will influence the ultimate composition of compounds on the skin surface at any given time. The distribution of these glands is not even across the body so it is highly likely that the profile of compounds sampled from the skin surface will be dependent upon body site.

1.3.2.3. Metabolism in skin

The skin is also a metabolically active organ and its structures contain a number of metabolic enzymes; results from histochemical tests indicate that the majority of skin enzymes are concentrated in the epidermis, hair follicles and sebaceous glands [86] [87]. Oxidation, reduction, hydrolysis, methylation and glucuronidation reactions have been reported to occur in skin. Concentrations of metabolic enzymes in skin tend to be low in relation to the liver however and these tend to have specific activities at less than 10% of those found in the liver [86]. In contrast esterase enzymes reportedly have high activity within the skin, topically applied benzoyl peroxide is completely hydrolysed to benzoic acid by human skin [71], indeed this property of human skin has been advantageously employed in transdermal drug delivery applications allowing the drug to be applied in a lipophillic form to cross the epidermis. Subsequent enzymatic activity within the skin converts it to a more hydrophilic compound to cross the dermis and enter the circulation [87].

Distribution of enzymes both within the layers of human skin and across the body is not well understood at this time. It has however been observed that topically applied drugs in *ex-vivo* skin from some areas will be completely metabolised, whereas the same drugs applied to skin from other body sites will be unaffected [87]. This provides further evidence for the variability in the profile of compounds likely to be observed across the skin of the human body.

Another form of metabolism that must be considered when discussing the chemical profile of human skin is one that occurs outside of the body; human skin supports microscopic flora and fauna which also metabolise compounds on the skin surface, whether endogenous or exogenous in origin. This facet of skin metabolism is most well-documented in the field of body odour research [84]. It has long been understood that, while sterile apocrine sweat is odourless, bacterial metabolism releases volatile odorous compounds from carriers creating ‘body odour’. Significant effort has been expended on understanding in this field and the bacteria responsible for metabolising known precursors to produce the principle causal molecules of malodour have been extensively reviewed, as have their distributions across the body surface [84]. The learning and observations derived from these body odour studies inform research and analytical studies that involve sampling from the surface of the human skin. Any compound

recovered from a skin sample may have been transported there as a conjugate with another molecule before being released during bacterial metabolism; yet one more pathway for transport across the skin.

1.3.3. Human skin analysis: current sampling techniques and key considerations

Early studies of the volatile constituents associated with human skin collected liquid sweat or used solvent extraction to obtain samples for analysis [99] [100]. More recently, headspace or direct contact sampling of the skin with a sorptive material have been described [88] [110] [111]. A number of continuous, on-line sampling and analysis techniques have also been applied [89] [90] [91] [92].

1.3.3.1. On-line sampling methods

The majority of sampling methods for collecting volatiles from human skin rely on liquid extraction or solvent or thermal desorption of the sampling medium, the resultant sample is then typically transferred to a gas chromatograph for subsequent separation and detection of volatile signatures [111]. Such approaches generate extensive VOC profiles that are rich in information, and are potentially useful for global profiling and non-targeted biomarker discovery. However, such analytical workflows are time consuming, and analysis times may not be applicable for clinical screening applications.

"Ambient" mass spectrometry approaches, where samples are introduced directly to an external ionisation source, have been applied to human skin to provide rapid and sensitive analyses. Mass spectral "fingerprints" obtained *in-vivo* directly from the skin surface with desorption electrospray ionisation (DESI) [89], secondary electrospray ionisation (SESI) [90] [91] and extractive electrospray ionisation (EESI) [92] are exciting and promising developments in this field that have yet to be applied to pilot metabolomic studies.

Enclosing a participants arm inside a Tedlar bag then directing the collected air for analysis by selected ion flow tube mass spectrometry (SIFT-MS) has provided real-time information for acetone emanating from skin as a potential indicator of blood glucose levels, further work may lead to techniques for monitoring type 1 diabetes [93].

Proton transfer reaction mass spectrometry (PTR-MS), in which gaseous samples are introduced by flowing synthetic air over the surface which is then directed to the a reaction region, has been applied to monitoring of UV-lipid peroxidation products of skin [94].

All these methods enable fast, selective and sensitive analysis of the area of a participant's skin presented to the instrument providing a near-real time, on-line analytical technique. These approaches are all non-invasive however these instruments are difficult to deploy for larger scale clinical studies:

- they are expensive and require skilled operators;
- they are difficult to sterilise and compliance with local biosecurity rules is often difficult;
- ambient ionisation techniques require the application of high voltages, presenting further challenges for clinical safety which clinicians may find off-putting;

Whilst offering a distinct time advantage, on-line analysis techniques require the participant to present the skin to be studied to the instrument, which is not always practical. Consider, for example, the practicalities of sampling and analysing skin from a large number of participants' over a short period. The difficulty increases if the study encompasses a large geographic region, or remote locations for which it would not be feasible to bring each participant to the instrument or move large instrumentation to such areas.

The current design of such sensitive analytical instrumentation is likely to prohibit their application at patients' bed-sides or in widespread metabolomic or screening studies until smaller units that require fewer resources have been developed.

Some efforts have been made to mimic canine olfaction by utilising electronic noses for monitoring volatile emissions from human skin, e-noses respond to volatile compounds using a sensor array and can be handheld instruments. One such study has employed an e-nose and demonstrated 87% accuracy in discriminating between patients with decompensated and compensated heart failure [95].

Near real-time monitoring of skin emissions has recently been achieved using ion mobility spectrometry (IMS) coupled with a short multi-capillary column in which the headspace from the skin surface was continually directed through a sampling loop the flow from which was then switched automatically to inject into the instrument with a sampling rate of 5 minutes [96]. Whilst IMS offers the advantages of being rapid, simple and sensitive the variation in instrumental configurations and the current lack of standardised substance libraries means that the identification of unknown compounds is difficult and the complex matrices expected from these kinds of samples may prove challenging in moving this technique forward without parallel GC-MS analysis.

1.3.3.2. Sweat collection methods

A common approach to sampling VOCs from the skin surface has been to collect sweat samples and a number of methods have been tested to this end.

Several simple sweat sampling techniques apply a form of cotton or gauze pad to collect samples, commonly from the axillae, usually with an exercise intervention or in a temperature controlled chamber to induce sweating. These pads can then be solvent extracted for liquid

injection into the analytical system or the headspace sampled onto an intermediate sorbent material such as a solid phase micro-extraction fiber (SPME) [88] or a tube packed with sorbent materials, typically porous polymers such as Tenax or Porapak Q [83].

These methods are likely to extract some compounds that are not volatile at naturally occurring body temperatures and the potential for transfer losses is increased with the two step analysis process from the sample pad to extraction solvent or secondary sorbent, and then recovery from these into the analytical instrument. One such study found common sterilisation techniques such as autoclave to not produce 'analytically clean' gauze pads even though they are considered biologically sterile [97].

Several methods for collecting sweat by encapsulating a limb or the entire body have been reported [98] [99]. The 'arm-bag method' is rather self explanatory. In this study sweat is collected in a polythene bag attached to the participants arm [99]. Whole body sweat collection has been attempted to remove variability between body sites and give a measure of the total electrolyte loss during exercise. In one study subjects were required to wash with distilled water and use an exercise bike inside a polythene bag for a set time period [98]. After exercise the subject washed again with distilled water whilst still inside the bag and the contents analysed by ion chromatography. This method was designed to ensure that no extraneous contaminants were sampled and therefore any results could only be a result of the individual's physiological state. Encapsulation of the whole body is designed to reduce variability between body sites however a great deal of information is lost by removing this variable. The experimental design of washing then protecting the skin from the environment so that only physiological factors are monitored may have application to other skin sampling techniques. The whole body sweat collection method claims to have an advantage over studies in which only one body area is sampled, for example the arm-bag method described previously as enclosing a limb causes water-logging inside the vessel used which results in sweat remaining on the skin, prevented from evaporating naturally by the surrounding air becoming saturated with water.

Many other methods have been employed to induce sweating in order to collect a sufficient sample volume [82]. Although these may be effective in increasing the rate of sample collection, they may serve to simply dilute an already low concentration sample and the biological and metabolic relevance of the collected sample must be called in to question. For a metabolomic or clinical application, sampling should cause minimal disruption to the participant's metabolic state and the high variability in sweat production, both inter and intra individual severely limits this sample type for this application.

1.3.3.3. Solvent extraction methods

Skin emanations have been sampled by directly washing the skin with organic solvents; one such method involves a glass funnel pressed against the skin surface to hold the solvent on the skin surface. The solvent and extracted compounds are then recovered using a Pasteur pipette and either analysed directly or pre-concentrated by drying on a rotary evaporator [100].

Several concerns with this approach as a solution to non-invasive sampling of biological VOCs immediately present themselves; the application of organic solvents to a participant's skin must be considered invasive and is highly likely to cause irritation. This technique is likely to extract a number of high molecular weight compounds along with the volatile of interest. By nature this technique will dilute the sample limiting the sensitivity and pre-concentration by drying is likely to cause major losses of the most volatile compounds extracted – something the authors observed when comparing to headspace samples from the same experiment [100].

Squalene monohydroperoxide (SQ-OOH), a product of sunlight-induced oxidative stress on the skin lipid squalene, has been sampled by wiping acetone-wet cotton pads across the skin surface. The sampled cotton pads are then solvent extracted, again diluting the collected sample, and analysed [101].

In summary solvent extraction techniques are inherently invasive, produce dilute samples, introduce contamination into the protocol, perturb the underlying metabolism that is being studied and require extensive sample work-up before analysis. This sampling approach has no place in non-invasive screening techniques.

1.3.3.4. Headspace sampling methods

Early headspace sampling methods relied on cotton pads, or even articles of clothing such as socks, to collect VOCs from the skin surfaces. These samples would then be sealed into a container, sometimes purged with clean carrier gas, and an adsorbent material was then exposed to the headspace. Analytes were then recovered by solvent or thermal desorption.

The same drawbacks are true for this sampling mode as with sampling sweat using absorbent pads. Many of the more recent studies into volatile emanations from human skin have used techniques sampling 'skin gas', or the headspace, directly above the skin surface; these offer a significant advantage in that only those compounds that are volatile at body temperature will reach the sampling medium.

For these headspace sampling protocols a headspace must first be created over the area of skin to be sampled and this has generally been achieved using a glass vial, funnel, petri-dish or even by inserting an entire arm into a jar or Tedlar bag. An adsorbent material is then exposed to the headspace after a certain equilibration time for sampling to commence.

One such sampler was designed to monitor medically significant VOCs such as acetone and acetaldehyde emitted from human skin and was based on passive flux sampling originally developed for material emissions applications [102]. A stainless steel petri-dish placed on the skin surface and sealed with a polytetrafluoroethylene (PTFE) o-ring created a headspace. Skin gas emanations moved through the open face of the sampler towards a trapping filter. In this case the trapping filter was activated with the derivatising agent 2,4-Dinitrophenylhydrazine (DNPH) which converts the carbonyl groups associated with aldehydes and ketones to hydrazone derivatives for analysis by high performance liquid chromatography (HPLC) [102].

One non-contact skin sampling method chose to enclose their volunteer's entire bodies, excepting the head, inside aluminised plastic bags which were then purged with filtered air onto sorbent tubes containing Porapak Q to trap the volatile emanations for subsequent solvent desorption and GC analysis [103]. Other sampling systems have used specialised bags to create a sealed headspace around limbs or appendages from which the headspace can be directed onto a 'cold trapping' device [104], or a sorbent material exposed directly into the enclosed bag for collection and preconcentration of volatile species [83]. Sampling in this manner has some key drawbacks; enclosing the body or a limb in an airtight bag prevents natural ventilation of the skin surface and hence will affect the physical chemical processes governing release into the headspace. Over-time the vapour around the skin is likely to become saturated with water, restricting the release of compounds both into the skin gas and onto the skin surface itself. This water vapour will condense on the inside surfaces of the sampling bag and can significantly effect recovery of a number compound types – a problem more commonly reported in exhaled breath analysis. A study into the feasibility of Tedlar bags for breath sampling demonstrated losses of a number of polar species including acetone, methanol, acetaldehyde and isoprene and some heavier compounds such as styrene [105].

Many successful non-contact skin sampling techniques have combined sampling and pre-concentration steps by exposing a sorptive material to a headspace created by a vial, petri dish or jar held against the skin surface; many of these have employed solid phase microextraction (SPME) fibres for sampling and enrichment. SPME offers the advantages of simple sensitive sampling and direct, solvent free analyte recovery by thermal desorption into a GC injector for analysis. SPME has been employed in profiling volatile emissions from the skin of the forearm [44], back [100], hand [44] and foot [106] predominantly in studies on malodour and principally polydimethylsiloxane (PDMS) – based fibres have been employed.

Whilst SPME is a widely used technique in this area application is limited by sensitivity due to the small volume of phase available, in response to this other samplers based on the same solid sorptive extraction mechanism utilising thin films of PDMS as the absorbent have been

introduced for headspace sampling [43] [107]. These sampling approaches offer much improved efficiency and sensitivity compared to SPME as they have a much higher surface area / extraction phase volume ratio, the physical chemical modelling of this will be explained in more detail in the following chapter [107].

These methods offer simple, non-invasive methodologies for sampling from human skin. The main difficulty in applying this kind of sampler to more widespread studies lies in creating the headspace – finding vials or jars to cover a representative portion of individuals of different sizes is likely to be challenging and with sampling times up to several hours ideally a sampler should not prohibit movement of the participant.

Recently, in response to this challenge, a new method for obtaining a headspace skin sample based on a thin film of PDMS has been developed; the PDMS is sandwiched between two layers of thin, flexible stainless steel mesh [45]. The bottom mesh prevents the sampler from coming into direct contact with the skin and the top to hold the sampler in place, now with this small, unobstrusive sampler the participant is free to move without being restricted by the sampling device. This technique was demonstrated to be reproducible for the compounds studied and showed potential for quantitative methods in future studies. One issue not addressed by the authors for this sampler is the inertness of the stainless steel meshes used to separate the membrane from the skin surface, a number of known malodour compounds have been reported to be labile and inert coated flow paths are necessary for the successful collection and thermal desorption of such compounds [108] [109].

1.3.3.5. Direct contact sampling methods

Perhaps the most straightforward of all techniques currently applied to skin analysis recovers analytes from skin by direct contact with a sampling medium. This has evolved beyond the use of fabrics and gauzes to solid sorptive materials. The sorptive material in contact with the skin surface absorbs or adsorbs a portion of the lipid layer, utilising the natural pre-concentration of VOCs in this layer, which contains a representative mixture of VOCs from the surface and provides additional storage stability [110]. Once samples have been collected, no further treatment is generally required for these samplers; they can simply be thermally desorbed in a heated GC injector or dedicated thermal desorption unit for analysis by GC.

A major problem associated with sampling human emanations is how to manage the large amount of aqueous eccrine perspiration that contains little metabolically relevant information; loading of water onto capillary columns most commonly employed in GC can damage the stationary phase, causing losses in sensitivity and peak shape, shifting retention times and poor reproducibility [39]. This was recognised by Bernier *et al* who studied mosquito attractants

in human VOC profiles using glass beads which are able to collect and concentrate non-aqueous emanations while minimising the collection of aqueous perspiration [39] [40]. The glass beads can then be placed directly into the injection port of a GC and thermally desorbed for analysis. A simple, easy and elegant sampling device this technique is limited in sensitivity by the relatively low affinity of the sampling phase [45] as the low concentration skin volatile emissions require an enrichment step before analysis .

To overcome this several sampling methods based on sorptive materials typically employed in GC column stationary phases have been developed for direct contact sampling of volatiles from human skin. As described for headspace sampling the most popular absorbent used in this field is PDMS. This safe, inert and flexible polymer can be formed into a number of configurations to be compatible with direct sampling of human skin.

SPME fibres have been stroked across the skin surface to sample feet and given results comparable to more classical headspace SPME sampling. This method is also limited in sensitivity by the low phase volumes and surface area / volume ratio [95].

Combining the high surface area of the glass beads and the higher affinity sorptive phase a PDMS coated stir-bar was developed for direct contact sampling of a pre-defined area of skin. A bespoke holder allowed the stir bar to be rolled across the skin surface and semi-quantitiative analysis has been achieved by embedding the stir bar with internal standards prior to sampling [41] [110]. This method of sampling VOCs from human skin has shown significant promise as a simple, non-invasive sampler. The main drawbacks with this sampler are difficulties associated with transfer handling reported due to the cylindrical geometry [45] and the need for continual rolling which means that realistically only one sample could be taken at any one time, unless several clinicians or researchers were available to sample every individual. Should participants be required to self administer the number of potential sampling locations is limited to where the participant can reach and how long someone can realistically be expected to reproducibly roll the sampler across their skin.

The principle geometry used in direct contact sampling from human skin with PDMS has been a thin film that conforms to the contours of the body giving a comfortable, simple, secure sampler with the desired high surface area to phase volume ratio for maximum extraction efficiency [41] [45] [110] [111]. A sorptive tape extractor (STE) has been employed to monitor perfume release from human skin [41] and the non-invasive identification of biomarkers from chronic wounds has been achieved using a thin film of PDMS known as the ‘Skin Patch’ [34] both of which simply require a PDMS film to be affixed to the skin surface for a defined time period. Skin Patches or STEs can be transferred directly to empty tubes compatible with

commercially available thermal desorption instrumentation for storage and subsequent analysis.

Direct contact samplers such as those described here are directly compatible with commercially available analytical systems and these PDMS based sampling approaches, headspace or direct contact, have generally been analysed by a two-stage desorption process in which volatiles released from the bulk sorbent in hundreds of millilitres of carrier gas are re-focussed, either by cryofocussing or cold trapping, to provide a second pre-concentration stage before GC analysis providing significant enhancements in sensitivity.

Direct contact skin sampling is non-invasive, pain-free, simple, self-administrable, portable and clean making it highly suitable for in-field or in-clinic sampling. Studies on the analytical utility of the Skin Patch have demonstrated it to be reproducible, with %RSDs from a minimum of 7% *in-vitro* to a maximum of 32% *in-vivo* of trace level endogenous VOCs, robust and sensitive [111]. It is possible to take multiple samples simultaneously, whether to study different emanation sites or to obtain duplicate samples, and the small sample footprint and ease of storage and transport make the Skin Patch approach highly suitable to wide spread non-invasive sampling for clinical or metabolomic applications.

To date direct contact skin sampling has been applied to a number of *ad hoc* studies exploring VOC profiles for monitoring perfume release [43], studying mosquito attractants [39] [40], examining fingerprints in human body odour, gender specific emanations [41], tracking the release of known metabolites [45] and identifying biomarkers of chronic wounds [34]. This thesis will further explore the potential for the Skin Patch sampler when applied to pilot metabolomic studies.

1.3.4. Skin sampling summary and key considerations

Previous research reports have shown the skin VOC profile to be highly variable and individualised with the potential to contain metabolically significant information about an individual. The identities of compounds sampled from the skin have varied significantly between studies and much of this variation can be attributed to the sampling techniques, as described previously. It is also apparent that the mechanical properties and distributions of glands and hair follicles will also have a significant effect on the class of compound recovered from a given sampling site. This was demonstrated well in a SPME based study investigating VOC profiles from the back and forearm of a group of participants; who noted 9 VOCs found only on the back as well as a higher level of exogenous compounds from personal care products [100]. These results correlate with another recent study sampling the upper back, forearm and thigh which also showed the upper back to yield the richest VOC profile, a fact that

the authors attribute to the high sebaceous gland density on the back when compared to the other sites [45].

In-vivo metabolic fingerprinting of human skin using extractive electrospray sampling has shown distinct fingerprints associated with the abdomen, foot and forehead of an individual; glucose was detected on the forehead but no other investigated body sites, whilst the foot sample gave a much less rich fingerprint leading the authors to propose that 'hard' foot skin allows less excretion of metabolites across it than softer skin on other body sites [92]. While these studies do not offer any particular direction for choice of sampling site they highlight the importance of carefully considering sampling site for skin studies and may explain the difference in identified compounds between studies.

Studies on age profiling with skin VOCs have found a higher prevalence of exogeneous compounds hexyl salicylate and α-hexyl cinnamaldehyde in younger subjects (<39 years) and attribute this to a more frequent use of personal care products in younger people while dimethylsulphone, benzothiazole and nonanal were proposed as 'biomarkers of increased age'[100]. Interestingly another study investigating the effect of age on VOC profiles identified 2-nonenal as the 'odour of old age' in a cohort of Japanese men [42]. The discrepancy between these two studies could be attributed to different sampling techniques, different sampling sites or perhaps a cultural phenomeneon due to the different climate and diet between the two cohorts [42] [100].

The different studies described here were chosen to illustrate the potential confounding factors associated with sampling the skin surface and highlight that the choice of sampling site, sampling technique, environment, diet and personal care product usage of the participants must have an effect on the data acquired.

Skin pre-treatment has been another highly variable factor between studies in the literature. In the days or hours prior to sampling, participants have been given specific instructions relating to diet, e.g no spicy food or garlic [83], personal care, e.g use of only unfragranced products and no deodorants or antiperspirants [9] [97], and exercise, in an attempt to restrict the confounding factors arising from exogenous compounds.

Immediately prior to sampling a number of protocols require a washing step to remove exogenous contaminants and sample only those emanating during the sampling period, typically clean water or soaps have been used for this purpose [88] [100] [111]. It is apparent that, whilst skin sampling offers an attractive simple non-invasive biological VOC sampling solution, careful consideration of participant cohorts, sampling site and pre-treatment techniques or restrictions must be undertaken, or at least appreciated, for a successful study.

1.4. THE ANALYSIS OF HUMAN SALIVA

In general healthy adults produce 500-1500 cm^3 of saliva per day at an approximate rate of 0.5 cm^3 min^{-1} [112]. A most valuable bio-fluid, saliva is critical to the preservation and maintenance of oral health in lubrication and protection, buffering and clearance, maintaining tooth integrity, antibacterial activity and taste and digestion [113]. Human saliva is a highly variable and individualised biological fluid comprised of the secretions from a number of salivary glands, gingival crevicular fluid, bacteria and their metabolites, epithelial cells and food debris [113].

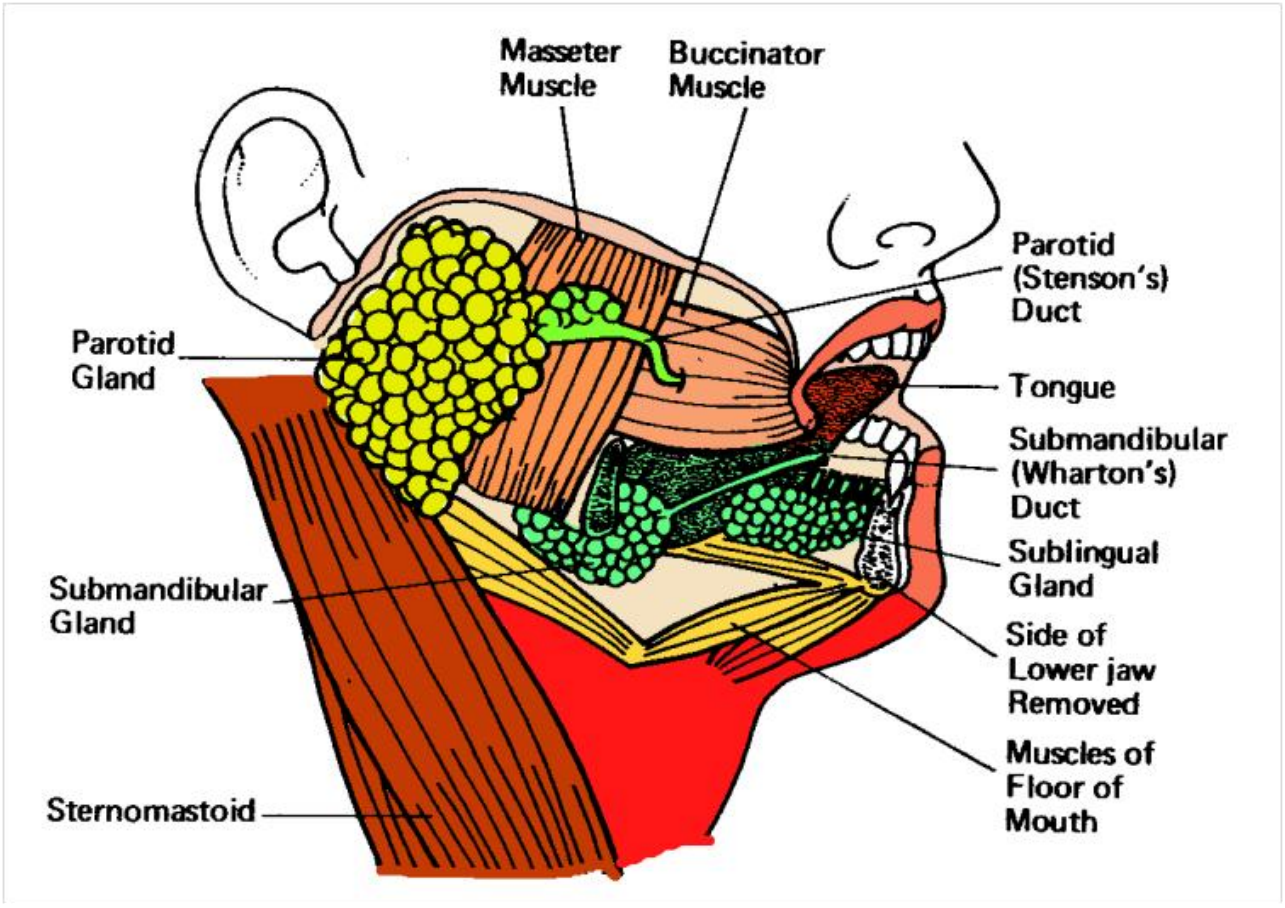


Figure 1.3 **Location of the major salivary glands in the human oral cavity. Reproduced from reference 114**

Figure 1.3 shows a cross section of the human oral cavity with the three major salivary glands labelled; paired parotid glands located opposite the maxillary first molars and the submandibular and sublingual glands, both located on the floor of the mouth, under the tongue. The major glands contribute the largest volume to whole saliva yet the composition will also be affected by secretions from minor salivary glands located on the lower lip, tongue, palate, cheeks and pharynx as well as gingival crevicular fluid, bacteria and their metabolites,

epithelial cells and food debris. The salivary glands consist of three cell types, acinar cells are the site of the 1st secretion and it is here that the type of secretion is determined. The secretion from each type of salivary gland varies and is determined by the ratio of serous to mucous glandular cells. Serous cells are located in the parotid and submandibular glands as well as those of the palate and the lingual glands, these secrete a watery fluid devoid of mucus, whereas mucus cells secrete a rich viscous solution and are found in the submandibular and sublingual glands as well as the lips and palate.

The second type of glandular cells are the duct system cells which can be further classified into three sections; intercalated duct system cells are the first in the duct network and connect secretions from the acinar cells to the rest of the gland, striated cells are the next in the network and it is here that reabsorption of sodium occurs for electrolyte regulation before the fluid reaches the final set of duct system cells; the excretory cells.

The final type of salivary gland cells are myoepithelial cells. These are elongated cell structures wrapped around acinar cells. On stimulation by neural processes the myoepithelial cells contract to constrict the acinar cells and secrete the fluid accumulated within.

VOCs in saliva may also be derived from serum, blood, skin lipids and microorganisms. The composition of whole saliva varies with circadian rhythm as well as a number of physiological and pathological conditions including: external stimuli such as taste and smell, metabolic influences associated with age, menstrual cycle, physical exercise, emotional state and drugs [113]. Quantitative analysis of saliva specimens has indicated meaningful correlations between circulating un-bound plasmatic and salivary levels of analytes [22] [59] [115]. This combined with the ready availability and non-invasive nature of the saliva sample make it a highly attractive alternative to blood and urine for screening human subjects [47].

1.4.1. Transport mechanisms: From circulating blood to saliva

While a large number of compounds in saliva are produced in the salivary glands the most biologically relevant information is likely to be found in the portion that passes directly from circulating blood vessels. The physical chemistry governing these processes is analogous to that described for transport across skin in Section 1.3 and an overview will be included here.

1.4.1.1. Passive diffusion

The capillaries surrounding salivary glands are relatively porous for many small molecules and passive diffusion is the most common route for migration of volatile compounds from blood to saliva [116]. To pass from serum to saliva by diffusion a molecule must cross the capillary wall, interstitial space and the membranes and cytoplasm of the cell types in the saliva gland. The ease with which a compound can passively diffuse from blood to saliva depends on its size and

polarity; lipophillic and neutral molecules pass from blood to saliva more efficiently than hydrophilic and ionised molecules as cell membranes are composed of phospholipids. Any compounds circulating as bound complexes are too large to cross into saliva by this route [113] [116].

1.4.1.2. Ultrafiltration

An extracellular mechanism ultrafiltration involves relatively small compounds filtering through the gaps between acinus and ductal cells to cross from blood vessels to saliva. Only compounds less than 1900 Da can cross in this way and their salivary concentrations are 300-3000 times lower than plasma [116].

1.4.1.3. Other minor mechanisms

Other mechanisms for transport into saliva from blood include active transport through the secretory cells of the glands and transudation from crevicular fluid or directly from the oral mucosa. Figure 1.4 shows a schematic representation of the modes of transport between blood and saliva.

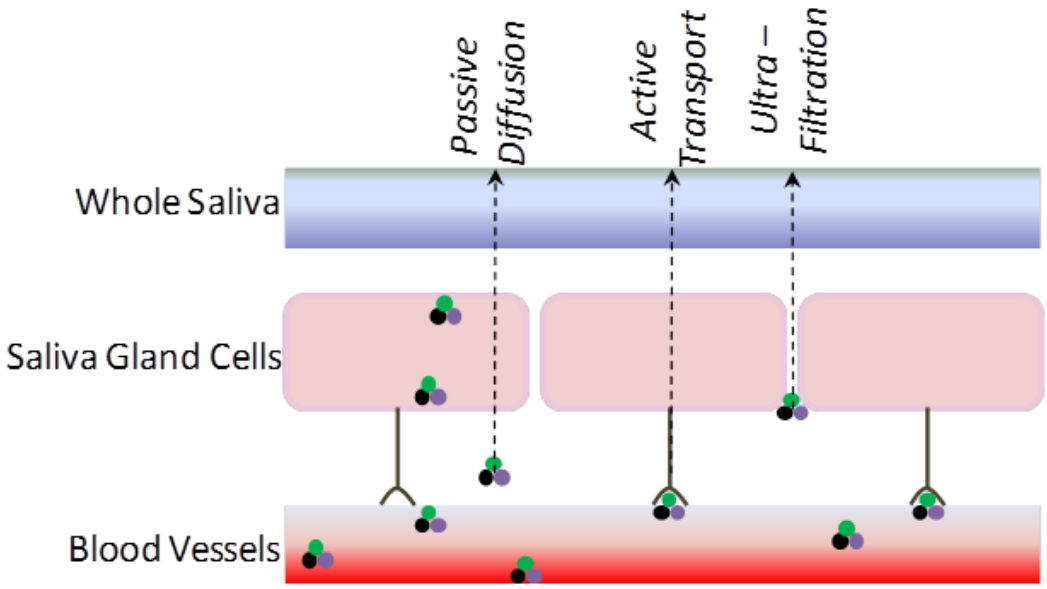


Figure 1.4 Schematic representation of the transport of biomolecules from blood capillaries into saliva. Adapted from reference 116.

1.4.2. The analysis of human saliva: Current sampling techniques and key considerations

Many protocols have been proposed for the collection of saliva samples. Bespoke methods have been described to collect the specific excretions from individual salivary glands which allow comparison between gland secretions. Such approaches require high-levels of specialist training to enable reliable sampling. The most common approach, as it is practical and

straightforward to achieve compliance with the sampling protocol, is to collect whole saliva. Even so, this approach requires carefully framed and rigorously conducted protocols to ensure consistency between samples and subjects [47].

Salivation can be induced using mastication or citric acid, known as a stimulated method. Alternatively participants may be asked to spit into a vial or sit quietly while saliva drains off their lower lip into a collection vessel, a technique known as passive drool, such approaches are termed unstimulated methods [117]. Introduction of citric acid to stimulate saliva lowers the pH and will alter the transport properties of some analytes, testosterone for example, and so the most commonly used method for collection of stimulated saliva uses the Salivette [117]. The Salivette is a cotton roll that the participant chews until it is saturated, after which the sample is recovered by centrifugation. The use of cotton wool introduces artefacts into the analysis and has been reported to enhance or reduce the results of immunoassays [117].

A study comparing bacterial levels in unstimulated samples obtained from spitting and passive drool reported 14-fold more bacteria in specimens obtained from spitting than those obtained by passive drool [117]. Bacterial action within a saliva sample is a vital factor operating from the moment sampling starts, hence the passive drool approach is commonly adopted to minimise such effects [118].

Standardisation of all saliva collection methods is a significant challenge and poor compliance with varied interpretations of sampling protocols has been reported [119]. It appears to be accepted that obtaining representative and reproducible whole saliva samples is a non-trivial task. Once collected, sample handling and storage procedures are also important with bacteria in the sample continuing to metabolise compounds and degrade the samples' integrity. Many salivary constituents have a short biological half-life, and rapidly degrade after sample collection. Further, the more volatile components are likely to be lost to the headspace or adsorbed/absorbed onto surfaces during collection and storage.

Storage procedures that specify freezing or refrigeration of saliva samples to stabilise them and inhibit bacterial action seek to address temperature instability and bacterial degradation on storage. One review of the salivary specimen as a new tool for investigation recommends aliquoting immediately on collection followed by storage at 4°C, -20°C or -80°C depending on the proposed storage time [117]. Other sample stabilisation steps include inhibition of enzyme or bacterial activity by snap freezing, or addition of inhibitors, denaturing agents or sodium azide for example [49] [117]. Such measures are time consuming, increase the number of steps in the analytical pathway, are susceptible to human error, while ensuring significant ventilation of the sample's headspace with subsequent loss of volatiles [47]. The resultant sampled material may well not be truly representative of the salivary composition at the moment of sampling [117]. The

challenges associated with reproducible collection and storage of saliva samples as well as the many and varied pre-treatment techniques necessary for a range of analysis techniques have been more extensively reviewed elsewhere [49] [56] [59] [113] [117] [120-122].

Many of these challenges have been addressed by adopting solid phase microextraction (SPME), for SPME offers simplicity, speed, reliability, and flexibility. Time-consuming pre-concentration is reproducibly achieved during sampling, and SPME integrates easily with GC instrumentation. Although SPME offers many improvements over established saliva sampling techniques for volatile components the challenges associated with standardisation of collection, stabilisation and storage of drooled saliva still exist [47].

1.5. CONCLUSIONS

This review has detailed the historical and anecdotal evidence for volatile emanations from humans containing biologically relevant information about an individual's state of health. Evidence for early identification of markers for systemic diseases in volatile profiles has been reviewed and the potential to harness these biological VOCs in new non-invasive, simple, fieldable sample techniques been explored.

Skin and saliva VOC profiles have been identified as biological compartments that lend themselves well to both non-invasive and large scale, portable sampling that may provide complementary information to the more established exhaled breath sample.

A review of the current sampling techniques for capturing VOC profiles from human skin has highlighted direct contact sampling with a sorptive material to offer advantages for simple, self-administrable, sensitive and robust sample collection. A number of key considerations for employing this kind of sample have been discussed including careful selection of sampling site, minimising confounding factors and exogenous contaminants and participant preparation. The PDMS based Skin Patch characterised by Riazanskaia *et. al.* has been selected to best fulfil the sampler requirements and will be evaluated for pilot metabolomic skin sampling in studies in this thesis.

The difficulties associated with collecting a representative saliva sample, briefly comprising techniques for inducing salivation, the relative increase in collected bacteria when saliva is collected by spitting versus drooling and the potential losses of volatile compounds during sampling have been reviewed and highlighted the number of potential confounding factors that require rigorous control in something apparently so simple as collecting an individual's saliva. When sample handling and storage techniques are also examined it becomes apparent that a wide range of factors can affect the VOC profile ultimately obtained from a saliva sample and the most volatile compounds are likely to be affected to a greater degree. This thesis will

extend the PDMS based sampling of the Skin Patch to saliva sampling and explore new avenues for collecting a more reliable saliva VOC sample.

1.6. THESIS

The underlying thesis of this work is that there is diagnostic information in volatile emanations from individuals in differing states of health and well-being that may be captured and exploited in developing the next generation of non-invasive clinical screening techniques.

The information contained in samples from different biological compartments may be comparable and will be complementary so different sample types may be applicable to different situations; these sample types can be incorporated into one workflow for sampling, analysis and data handling to produce complementary data and bring a level of rigor to metabolomic studies of volatile profiles, ultimately moving clinical diagnosis away from puncture wounds towards non-invasive methods.

1.7. RESEARCH OBJECTIVES

To test the central thesis a series of studies were designed around the following research objectives:

- Characterise and develop PDMS-based non-invasive sampling techniques for human skin and saliva.
- Critically evaluate and optimise existing workflows for pilot volatile metabolomic studies for the application to skin and saliva samples.
- Evaluate the performance of the samplers and workflows in pilot metabolomic studies with healthy young adults subject to a stress intervention.

For successful application of novel samplers to untargeted global metabolite profiling it is first necessary to appreciate the contribution of each step in a large multi-disciplinary work flow and the impact they may have on the study design. Chapter 2 describes the key elements that must be evaluated and optimised prior to designing a metabolomic study and discusses the relevant theory and key parameters.

2 AN INTRODUCTION TO VOC SAMPLING: A MULTIDISCIPLINARY COMBINATION OF ETHICS, *IN-VIVO* SAMPLING TECHNIQUES, ADVANCED INSTRUMENTATION AND ADVANCED DATA PROCESSING

2.1. INTRODUCTION

Global metabolite profiling of complex *in-vivo* samples requires a multi-disciplinary workflow that starts with ethical clearance for studies with human volunteers, encompasses the design of novel sampling techniques and the optimisation of analytical methods and ends with a rigorous adherence to data handling protocols. Currently such workflows are dynamic processes, with each study providing invaluable experience and insight. Objective evaluation of this must lead to modification of the work flow in an iterative process with each study enhancing the next.

This Chapter discusses the key elements that must be considered before undertaking a pilot metabolomic study and include critical factors in designing a non-invasive VOC sampling method, an overview of analytical instrumentation for the recovery, separation and detection of VOCs and finally the techniques available to handle and characterise complex data sets. This Chapter presents a working knowledge of the theories and a discussion of the most relevant principles for each stage of the analytical workflow, more in-depth theoretical explanations have been extensively reviewed elsewhere [123-127] and as such do not form part of this thesis.

2.2. ABSORPTIVE SAMPLING FOR *IN-VIVO* STUDIES

Polydimethylsiloxane (PDMS), Figure 2.1, a non-toxic, highly hydrophobic polymer was chosen for the sampling material for these studies and its application as an extraction medium in numerous geometric forms for liquid or gaseous samples has been reviewed for a wide range of sampling challenges [128]. Perhaps the first notable application of PDMS in analytical chemistry was as a stationary phase in gas-liquid chromatography [129] and since then its capabilities for sample enrichment have led to its use as a sampling medium for organic compounds from air, water, soil and biological matrices [130-133]. Unlike adsorptive sampling usually applied to these matrix types in which compounds are trapped by adsorption onto the sorbent surface, analytes partition into the bulk phase of the PDMS.

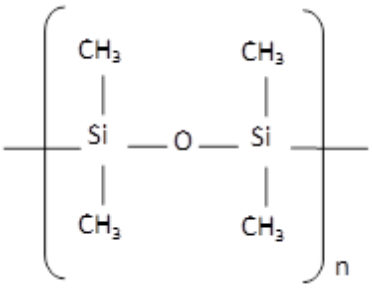


Figure 2.1 The structure of polydimethylsiloxane (PDMS).

The retention properties of PDMS are not influenced by the presence of high levels of water or other analytes as each solute has its own partitioning equilibrium into the PDMS, so its retention capabilities are largely independent of sample matrix [128].

PDMS is a non-toxic hydrophobic polymer which can be formed into a required shape or thickness in a simple and reproducible manner which lends it the versatility to be applied to a number of sampling challenges. The first exploration of PDMS as an enrichment phase for organic sampling used open tubular trapping (OTT). In this technique a layer of PDMS is coated onto a capillary wall, much like a gas chromatography column, and a liquid or gas sample is drawn through the tube. OTT found limited application as the sample capacity was limited by the thickness of the coating, so metres of tubing are required to give adequate limits of detection. In a similar fashion, larger tubes packed with PDMS particles have been successful for gas sampling; the sample gas passes through the tube and the analytes partition into the phase. This approach is hampered by the rapid breakthrough of the most volatile species [134].

Solid phase microextraction (SPME) often employs a PDMS phase coated on the outside of a needle; this needle is either placed directly into a sample or into the headspace above it, again this technique is somewhat limited by the volume of PDMS on the needle. Stir bar sorptive extraction (SBSE) and solid phase extraction (SPE) employ a layer of PDMS coated on the

outside of a magnetic stir bar or titanium support which is then immersed in the sample. This approach provides the enrichment factor of the PDMS tubes with a relatively large PDMS volume whilst maintaining the simplicity of SPME [134]. The use of thin films of PDMS, unsupported by needle or stir bar, has also been successful in this form of sampling and approaches such as thin film micro-extraction, sorptive tape extraction and the 'Skin Patch' have shown application in direct contact sampling with solid matrices [43] [34] [111] [135].
In adopting PDMS as a sampling medium there are three key processes to consider:

- absorption into (partitioning);
- diffusion through;
- and evaporation from the membrane (permeability).

If there is a difference in chemical potential of a species between two phases in contact there will be a net movement of that species in the direction to bring the system to equilibrium. For PDMS this is usually described by solubility and partitioning coefficients and is based on the assumption that partitioning coefficients between PDMS and sample matrix ($K_{PDMS/S}$) are approximately proportional to the standard partitioning coefficients between 1-octanol and water used for biological systems ($K_{O/W}$) [134], summarised in Equation 2.1.

Equation 2. 1

$$K_{O/W} = K_{PDMS/S} = \frac{C_{PDMS}}{C_s} = \frac{m_{PDMS}}{m_s} \times \frac{V_s}{V_{PDMS}}$$

Where C represents the concentration of analyte in the PDMS or sample matrix and m the mass in the same, V represents the volume of the PDMS or sample matrix and the ratio of the two can be described as the phase ratio β.

Equation 2. 2

$$\beta = \frac{V_s}{V_{PDMS}}$$

Entering β into Equation 2.1 then rearranging in terms of extraction efficiency we can now see that the mass of analyte extracted into the PDMS is proportional to the partition coefficient and inversely proportional to the phase ratio.

Equation 2. 3

$$\frac{m_{PDMS}}{m_s} = \frac{m_{PDMS}}{(m_{total-}m_{PDMS})} = \frac{K_{O/W}}{\beta}$$

The physical factors governing extraction efficiency of an analyte into PDMS are the phase-ratio and the partition coefficient. For *in-vivo* sampling common approaches to increasing $K_{O/W}$ such as raising the temperature, or pH control will not generally be possible, therefore when designing an *in-vivo* PDMS based sampler it is important to consider the phase ratio, β.

Absorption is an equilibrium process, when PDMS is exposed to a sample matrix the concentration in the polymer will initially rise rapidly in a linear fashion described as the kinetic region. A plot of time versus analyte concentration in PDMS (C_{PDMS}) would ultimately reach a plateau once the system is at equilibrium, at this point the extraction into the PDMS will equal the elimination from it [128]. At sampling time t, C_{PDMS} will be dependent on the concentration in the sample matrix, C_s, and the uptake and elimination constants k_1 and k_2 respectively.

Equation 2. 4

$$C_{PDMS} = C_S \frac{k_1}{k_2} [1 - e^{-k_2 t}]$$

Partitioning into a PDMS sampler depends on the phase ratio, the analyte's partition coefficient and the sampling time. For *in-vivo* sampling of biological systems only β and t are available parameters for optimisation and while increasing the thickness of a PDMS membrane in direct contact with a surface will decrease β greater extraction efficiency in a given procedure is more likely to be achieved by increasing the surface area in contact with the sample, in other words increasing the analyte flux into the membrane [131].

When sampling from a solid surface using a PDMS membrane the PDMS is not submerged in the sample, only one side is in contact, in these cases it is important to consider factors other than partitioning into the phase when selecting sampler parameters. In cases such as these there exists a concentration gradient across the membrane from the sample surface to the air on the non-sampling side of the PDMS. Sampling in this situation will begin following the partitioning mechanism already described then continue with diffusion across the membrane, along a concentration gradient from the inner surface to the outer and ultimately elimination from the other side. Such processes are utilised in techniques such as membrane inlet mass spectrometry as a method for selectively eliminating matrix interferences [136-138].

Under steady state conditions the flow rate of a given solute, I_{ss} (moles s^{-1}), across a membrane of area *A* (cm^2) and thickness x (cm) can be described in terms of the difference in concentration between the membrane surfaces, ΔC (moles cm^{-3}) and the diffusion, *D* (cm^2 s^{-1}), and partition, *P*, coefficients of the solute in the membrane:

Equation 2. 5

$$I_{ss} = \frac{ADP\Delta C}{x}$$

For a given solute *D* and *P* are constant and only ΔC, *A* and x may be manipulated to control I_{ss}. For *in-vivo* sampling ΔC is typically unknown and uncontrolled so analyte losses due to permeation across the PDMS membrane can be minimised by controlling the membrane thickness and surface area:

$$I_{ss} \propto \frac{A}{x}$$

Increasing *A* will increase the amount of solute partitioning into the PDMS at a given time, so increasing I_{ss}, whilst increasing x will decrease I_{ss} and reduce the loss of VOCs from the outer PDMS surface in a given time.

Figure 2.2 shows a simplified schematic describing absorption of one compound from the skin surface, onto the inner surface of the PDMS, diffusion through the membrane, absorption onto the outer surface of the PDMS and elimination into the diffusion layer of air above it. This system would ultimately reach equilibrium and the rate at which equilibrium is achieved will be compound specific and determined by the partition diffusion coefficients for the analyte.

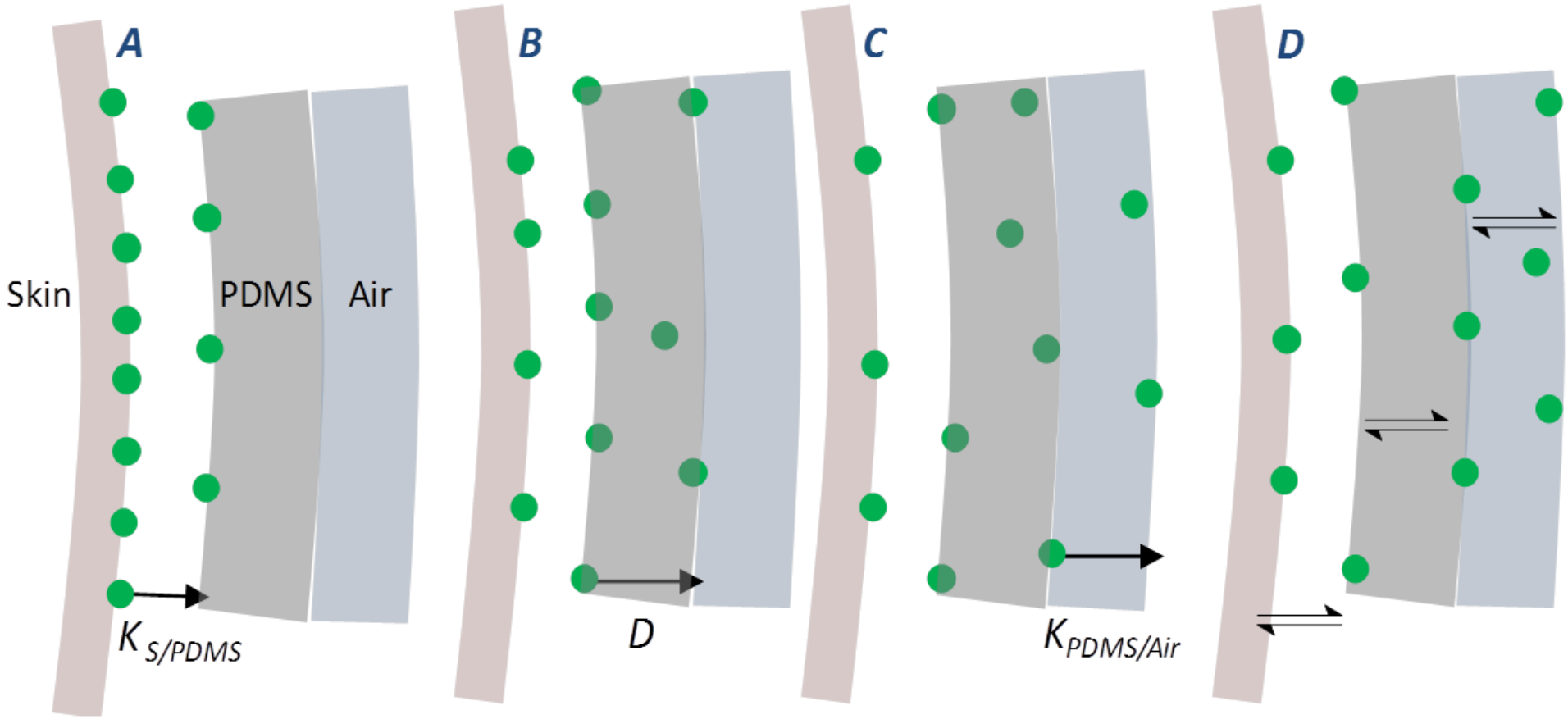


Figure 2.2 Simplified schematic diagram representing the partitioning and permeation processes governing absorptive sampling from the skin surface: A: Initially there is a concentration gradient from the skin to the inner PDMS surface causing a net movement of VOCs governed by the partition coefficient KS/PDMS. B: concentration gradient through the PDMS membrane from the inner to outer surface, diffusion occurs across the membrane with diffusion coefficient D. C: concentration gradient from the outer PDMS surface to the boundary layer of air above it, VOCs are eliminated from the PDMS with partitioning coefficient KPDMS/Air. D: Assuming a constant skin surface concentration these processes will reach equilibrium and a steady state CPDMS achieved: D. KS/PDMS, D and KPDMS/Air are compound dependant so the rate at which a steady state CPDMS is realised will vary with compound; a key consideration when optimising sampling protocols.

When sampling the skin surface using a PDMS membrane analyte uptake will be governed by partitioning into the PDMS, for which we can manipulate the phase ratio and sampling time and the permeability across the membrane, which can be limited by the path length and the sampling time. Optimal sampler conditions are likely to be based on a compromise between these factors.
pH and temperature are also factors that will contribute to the rate at which VOCs are absorbed and eliminated by the PDMS samplers. These factors are less practical to control for in-field *in-vivo* sampling and initial work will focus only on sampling time and the sampler dimensions.
These studies utilise a combination of SPE and thin film PDMS based sampling approaches for the recovery of VOCs from human skin and saliva. Specific sampler characteristics will be discussed in Sections 3.1 and 3.2.

2.3. EXTRACTION OF VOCs FROM PDMS SAMPLERS

There are two main techniques applied to recovering analytes from PDMS for analysis; solvent or thermal desorption. In brief solvent desorption (SD) involves extracting the PDMS with a suitable liquid solvent for injection into the analytical system whilst thermal desorption (TD) employs heat and a flow of inert carrier gas to transport desorbed volatiles to the analytical system.
The advantages of thermal over solvent desorption are reviewed in detail by Woolfenden in 'Gas Chromatography' and will be discussed in brief here [125]. Thermal desorption is a pre-concentration technique in which analytes from hundreds of litres of a vapour sample undergo two stages of focussing before transfer to the analytical column in ~100 µl of carrier gas, giving concentration enhancements up to 1 million fold. Conversely solvent extraction typically involves extraction of analytes into millilitres of solvent of which microlitres are then introduced into the analytical system. Thus solvent extraction acts to dilute the sample whereas thermal desorption acts as a concentrator.
The introduction of organic solvents into the analytical procedure brings sources of contamination; whilst it is common to find carrier gas cylinders with sub ppm level impurities in routine laboratories achieving the same level of purity in solvents is difficult and expensive. Once such solvents are opened within the laboratory they begin diffusively sampling laboratory air whilst evaporating into it to contaminate other solvents and it is difficult to maintain the level of solvent purity originally purchased.
The key consideration for direct desorption applications such as extraction of VOCs from PDMS samplers is that thermal desorption enables transfer of 100 % of recovered compounds to the

analytical system, whereas solvent desorption invariably allows transfer of only a portion of the resultant solution.

Whilst thermal desorption can be fully automated requiring no sample preparation steps, solvent desorption is more labour intensive, with greater potential for introducing operator error and exposes individuals to hazardous solvents, such as CS_2, which is commonly employed for such extractions. Thermal desorption is a dynamic extraction process and methods typically achieve greater than 95% recovery whereas many solvent desorption techniques employ a static extraction which limits recovery to approximately 75% [125].

With numerous analytical methods favouring the use of mass spectrometric detectors many extraction solvents can cause interferences such as baseline disturbances, signal quenching, overloading or masking peaks of interest. Thermal desorption on the other hand is inherently solvent free and artefacts exhibited by common sorbents tend to be in the order of nanograms, giving discreet peaks rather than overloading chromatographic systems as is often seen with solvent injections. Artefacts exhibited from the thermal desorption of PDMS are well characterised and not endogenous to humans so are unlikely to cause interferences in these studies.

A review of the above led to thermal desorption being chosen for analyte recovery in these studies, the PDMS Skin Patch or SPE-td cartridge was placed inside empty thermal desorption tubes for introduction to a Unity 2™ thermal desorber (Markes International, Llantrisant, UK).

2.3.1. Principles of thermal desorption

In its simplest form thermal desorption is an extension of gas-solid chromatography, a material or sorbent is heated in a flow of inert carrier gas and the desorbed VOCs are swept onto a gas chromatography column for subsequent separation and detection. As with gas chromatography key parameters are carrier flow rate, desorption temperature and desorption time.

Standard sorbent tubes contain hundreds of milligrams of sorbent and direct desorption applications, such as thermal desorption of PDMS, achieve exhaustive extraction via a dynamic equilibrium process. As such either of these techniques typically require tens of millilitres of carrier gas for complete desorption of the sorbent material [139]. Gas volumes of this magnitude are not compatible with standard gas chromatography columns and result in poor analyte focussing at the column head giving broad peaks, poor resolution and compromised signal to noise ratios.

A 2-stage desorption process during which analytes released from the sorbent tube onto an electrically cooled secondary focussing trap overcomes this limitation. Typically a ‘cold trap’ consists of a narrow bore quartz tube (2 mm id) packed with a 60 mm long sorbent bed held at a

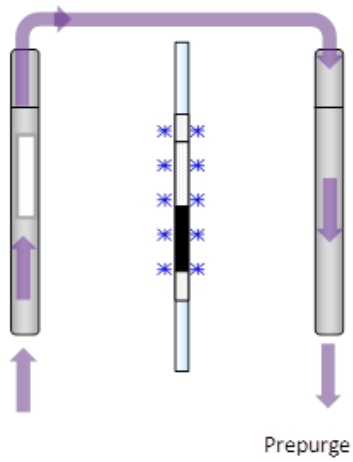
low temperature (-30 - +20 ºC). Desorbed analytes from the sorbent tube are focussed on the cold trap which is then heated rapidly, at up to 100ºC s^{-1}, transferring analytes to the GC column in a narrow band of vapour, typically 100 to 200 µl, yielding improved sensitivity and peak shape compared to single stage desorption [139].

Selection of sorbents for the cold trap is influenced by a number of factors. Principally for sampling using sorbent tubes it is advisable to choose a sorbent of the same or greater "strength" than those in the sample tube. For direct desorption of a PDMS membrane the choice is less limited and becomes a balance between several key factors including strength, hydrophobicity, inertness, artefact levels and thermal stability [125]. It is important to select a sorbent that not only quantitatively retains compounds of interest but that also releases them efficiently, so it is not recommended to simply use the strongest sorbent available. For profiling studies such as those explored in this thesis it is common to encounter a wide volatility range and the properties of more than one adsorbent may be required.

Thermal desorption is achieved using a 'backflush' mode to desorb the cold trap (Figure 2.3) which means that the VOCs enter the cold trap in a flow of gas which is then reversed to desorb them into the analytical system. This allows two or more adsorbent beds to be packed into the cold trap in series, extending the volatility range of VOCs that may be included in the analytical work-up of the sample. The desorbed VOCs enter the cold trap encountering the weakest adsorbent first where the less volatile compounds are retained, the more volatile compounds breakthrough the weak adsorbent bed into the stronger adsorbent where they are retained. If desorption of the cold trap adsorbent used a forward flow approach the VOCs would have to pass through the entire cold trap to reach the analytical system. With a multi-bed adsorbent trap this would require the least volatile and most active compounds to elute through a highly retentive strong adsorbent bed. This would require a large volume of gas and negate the benefits of using two stage desorption. The ability to backflush the cold trap allows the sample gas to leave the cold trap in the same direction as it entered [139] with the additional benefit that the desorption gas supply is protected from overly contaminated samples.

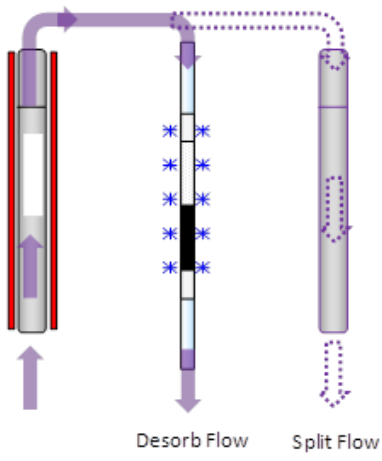
The system used in these studies, Unity 2 thermal desorption unit (Markes International, Llantrisant UK), performs an automatic leak test to ensure sample integrity before heating, a failure of the leak test will halt sample analysis. Figure 2.3 summarises the subsequent thermal desorption process described above and performed automatically by this system.

Prepurge



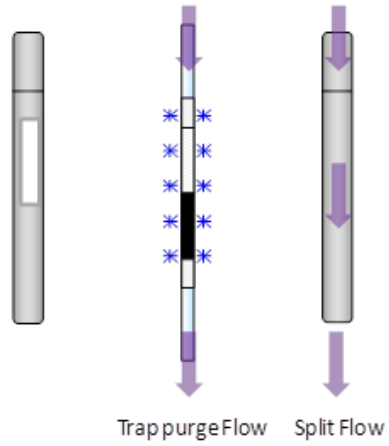
Prior to heating the sample tube the Unity performs an ambient temperature purge, this serves to selectively eliminate interferences such as oxygen and water by purging them to the split vent before heating the sample tube

Primary Desorption



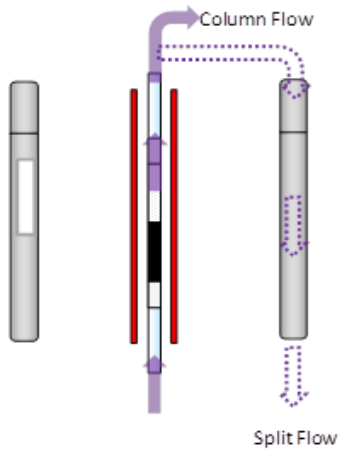
The sample tube is heated and a flow of inert carrier gas sweeps desorbed analytes onto the cold trap, held at low temperature, an optional split may be employed here.

Pre Trap Fire Purge



The cold trap and split filter are purged with a flow of inert carrier gas. This enables selective elimination of solvent, water and any permanent gases from the cold trap before it is ballistically heated during trap desorption.

Secondary Desorption



The cold trap is rapidly heated with the flow of inert carrier gas in the backflush direction, sweeping desorbed analytes onto the GC column. An optional split may be employed here.

Figure 2.3 A depiction of the stages of operation during thermal desorption of a PDMS Skin Patch held inside an empty stainless steel tube.

A two-bed cold trap containing Tenax TA, an inert, hydrophobic polymeric sorbent backed up with Carbograph 1TD a stronger, hydrophobic graphitised carbon black sorbent was specified for quantitative retention of compounds with boiling points in the range 30 – 500 °C whilst being virtually unaffected by high water content [140].

Successful application of thermal desorption to recover VOCs from PDMS samplers requires quantitative, and complete transfer of analytes from the sampler to the cold trap and the subsequent analytical systems. Key method parameters include the sample desorption temperature, flow rate of desorption carrier gas (desorb flow) and the time allowed for extraction. To ensure retention of the extracted analytes on the cold trap it is important to consider the breakthrough volumes of the most volatile compounds on the sorbents in the cold trap, at the temperature of the cold trap during sample desorption (cold trap low temperature).

Breakthrough is the term used to describe a situation in which an analyte is no longer quantitatively retained on a sorbent, the breakthrough volume is the volume of carrier or sample gas that can pass through the sorbent bed before breakthrough is achieved. There are extensive lists of published breakthrough volumes for a wide range of analytes on a wide range of sorbents; it is advisable to use these as a guideline when first designing a new analytical method. For profiling studies such as those undertaken on skin and saliva in this thesis where the sample compositions are unknown a literature search reveals acetic acid and acetone to be among the most volatile compounds recovered from skin and saliva using a PDMS based sampler [96] [100] [142]. These compounds have a breakthrough volume on a 60 mm long 200 mg bed of Tenax at 20 °C of 550 and 500 mL respectively [142]. When optimising the desorption of the PDMS sampler to ensure quantitative recovery of the sampled VOC it is also important to ensure that the most volatile compounds do not breakthrough the cold trap; method development and optimisation is likely to be a compromise between these two factors and is discussed further in Section 3.4.1.

2.4. SEPARATION OF VOLATILE MIXTURES

Gas chromatography (GC) is the gold standard technique for separating complex mixtures of VOCs and of the various iterations capillary GC is by far the most widely used today. Capillary GC passes a gas phase mixture through an open tubular column coated with an absorbing liquid polymer film, known as a stationary phase. The gas phase analytes are carried in a flow of inert carrier gas, known as the mobile phase, and separation of the volatile analytes is achieved through partitioning between these two phases.

2.4.1. Principles of gas chromatography

A solute passing through a GC column interacts with the stationary phase so that at a certain point the solute's net velocity is less than that of the mobile phase; the solute is retained on the stationary phase. Different solutes will distribute differently between the two phases and so will migrate through the column at different velocities, eluting and being detected at different times, these are known as retention times. This provides the basis of gas chromatographic separation and careful control of key parameters will govern the separation of compounds of interest [125].

Analysis of complex VOC mixtures by GC presents a significant challenge in selecting and optimising method parameters. The wide volatility range of unknown analytes means that there is unlikely to be one perfect method and compromises will be necessary.

The principle factor governing retention and separation of analytes by GC is the column itself; key column parameters include selection of the optimum stationary phase, length, film thickness and internal diameter. Selection of a stationary phase can be a difficult task for a profiling study from which a wide range of functionalities and volatilities are expected, key considerations include polarity, selectivity and bleed [143]. While it is advisable to select a stationary phase with similar characteristics to the analytes, it will always be difficult to match a column to volatile fatty acids and long chain hydrocarbons that are to be expected with skin samples. As a result of the wode range of compounds expected from skin samples in this study a general purpose 5% phenyl 95% dimethyl polysilxoane stationary phase was chosen which exhibits low polarity, low bleed and has a high temperature limit [143].

Other column parameters affecting separation include internal diameter (i.d), length and film thickness. A larger internal diameter (i.d) column will allow a higher column flow, this can be advantageous when analysing less volatile more 'sticky' compounds and is particularly important when coupled with thermal desorption operating in splitless mode where the cold trap desorption flow is equal to the column flow and it is desirable to have as high a column flow as possible for efficient desorption. Column efficiency, however, is inversely proportional to internal diameter so where separation is poor a smaller i.d column can improve resolution. Smaller i.d columns also have a more limited capacity so the choice of column diameter is a balance between desired flow rates, separation by the stationary phase and the expected column loading [125] [143].

A longer GC column will offer improved efficiency with the payoff of longer analysis times and higher levels of column bleed. For complex samples with a high number of solutes a long column can be advantageous. A thicker film column, that is to say a higher volume of stationary phase, will offer increased retention at a given temperature than a thinner film; this can usefully be applied to

improving retention of the most volatile compounds in a mixture, conversely thinner films may be employed to allow highly retained compounds to elute faster [143].

For these studies a 60 m long column with a film thickness of 0.25 µm and an internal diameter of 0.25 mm was chosen for profiling unknowns over wide volatility range, representing a compromise between retention of the most volatile and timely elution of the least volatile compounds, loading capacity and separation efficiency.

Other factors affecting GC separation include temperature and mobile phase flow rate. Simply put higher temperatures and higher flow rates reduce analysis time and separation so selection of these factors is a balance between achieving sufficient separation within a practical analysis time. Slower heating rates again yield improved separation, indeed many GC methods use a constant temperature (isothermal) for maximum separation. For complex mixtures over a wide volatility range the time necessary for an isothermal method would be prohibitive so a temperature programmed method must be employed in which the column temperature is increased across the chromatographic run to speed up elution of the less volatile compounds. Selection of flow rates and temperature programmes will be discussed in more detail in Section 3.4.2.

2.5. DETECTORS FOR GAS CHROMATOGRAPHY

Gas chromatography has a distinct advantage over many other common separation techniques in that it can be coupled to a wide range of detectors. These fall neatly into two categories; selective detectors which detect only specific classes of compounds based on a molecular or physical property for example an NPD detector which responds to nitrogen or phosphorous, or universal detectors which are able to detect all (or most) compounds eluting from the GC. For profiling studies in which a wide range of unknown compounds are of interest the choice is simple; the only option is a universal detector. Examples of universal detectors include the flame ionisation detector (FID), a mass sensitive detector which gives a univariate response based on the mass of carbon detected, the thermal conductivity detector (TCD), another univariate detector which responds to anything with a different thermal conductivity from the carrier gas and the mass spectrometer (MS), a mass sensitive, multivariate detector which not only responds to almost any compound but also gives structural information making the mass spectrometer the detector of choice for profiling of unknown mixtures [139].

First coupled to GC by McLafferty and Gohlke in the 1950's, mass spectrometry enjoys a rich heritage dating back to J.J.Thompson's experiments on the characterisation of electrons during which he determined their *mass to charge* ratio and went on to develop the first mass

spectrometer in 1912. Since then there have been numerous and wide – ranging advances in this technique to allow the introduction of gas, liquid or solid samples at vacuum or ambient pressure with many different mass analysers with varying properties available to suit an array of applications. Extensive literature resources are available detailing and describing every aspect of mass spectrometry in detail [126], the descriptions in this section will therefore be limited only to those techniques employed in this work.

2.5.1. Principles of mass spectrometry

Mass spectrometry is a technique in which gas phase ions are formed and separated in space or time and detected according to their mass-to-charge ratio, conventionally denoted as *m/z.* Schematically, a mass spectrometer consists of a sample introduction system – in this case the GC – an ionisation source, a mass analyser and a detector (Figure 2.4).

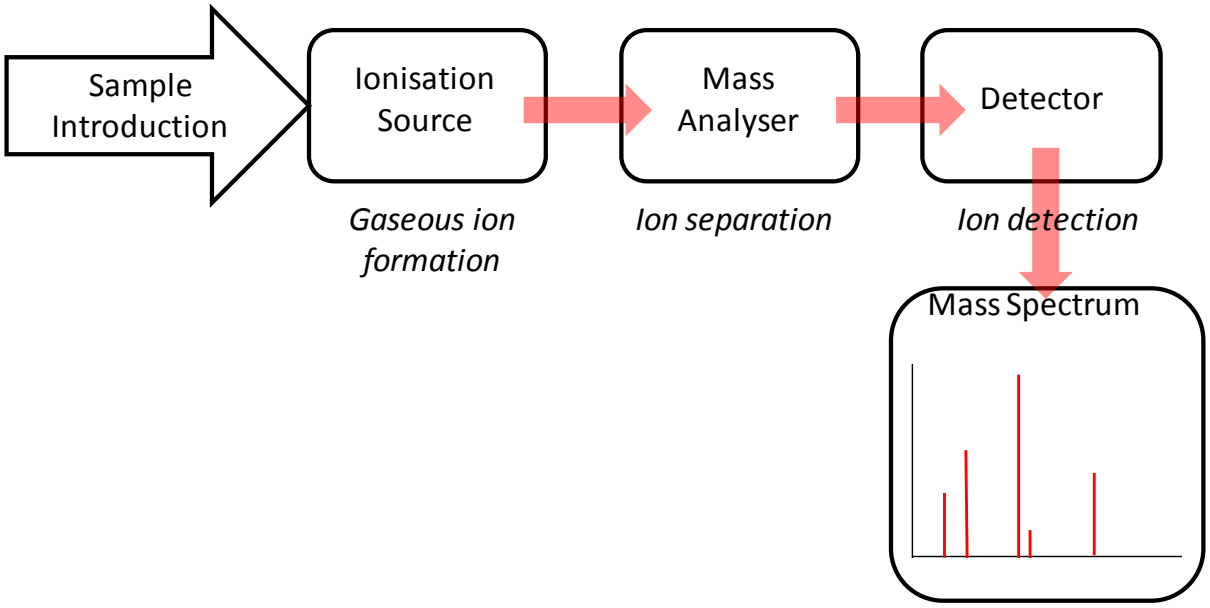


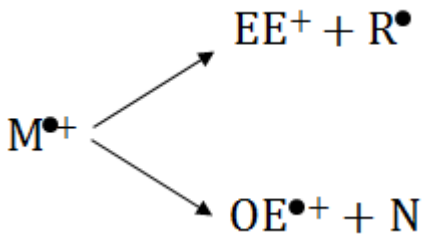
Figure 2.4 Simplified schematic diagram of a mass spectrometer

2.5.1.1. Electron ionisation

When coupled to a gas chromatograph the analytes arrive at the mass spectrometer as separated peaks of gaseous molecules and the first stage is to ionise them. Electron ionisation, first devised by Dempster in 1918, accelerates electrons generated by a hot rhenium filament towards an anode (trap) perpendicular to the flow of sample gas, these electrons collide with neutral analyte molecules, electronic excitations occur and, where there is sufficient energy, an electron can be expelled [144]. Figure 2.5 shows a schematic of an EI source.

$$M + e^- \rightarrow M^{\bullet+} + 2e^-$$ Scheme 2.1

Where M is the neutral analyte molecule and e^- an electron. This technique is commonly applied to organic compounds and is a high energy technique which produces not only ionised parent molecules but often also breaks them apart into fragments. A curve of number of ions formed against electron energy (the kinetic energy supplied to the electrons) gives a wide maximum at 70 electron volts (eV) for organic molecules and is the standard value used for electron ionisation mass spectrometry with gas chromatography. The energy transferred to the molecule during ionisation often exceeds the ionisation energy and so the radical cation formed is likely to undergo extensive fragmentation to form either a radical ($R^{\bullet}$) and an ion with an even number of electrons (EE), or a neutral molecule (N) and a new radical cation with an odd number of electrons (OE) [126]:



Scheme 2. 2

These primary product ions can undergo several further fragmentations and the resultant ions are collimated into the mass analyser by a repeller electrode and electrostatic focussing lenses where they are separated based on their mass-to-charge ratio and detected in proportion to their abundance, producing the mass spectrum of a molecule.

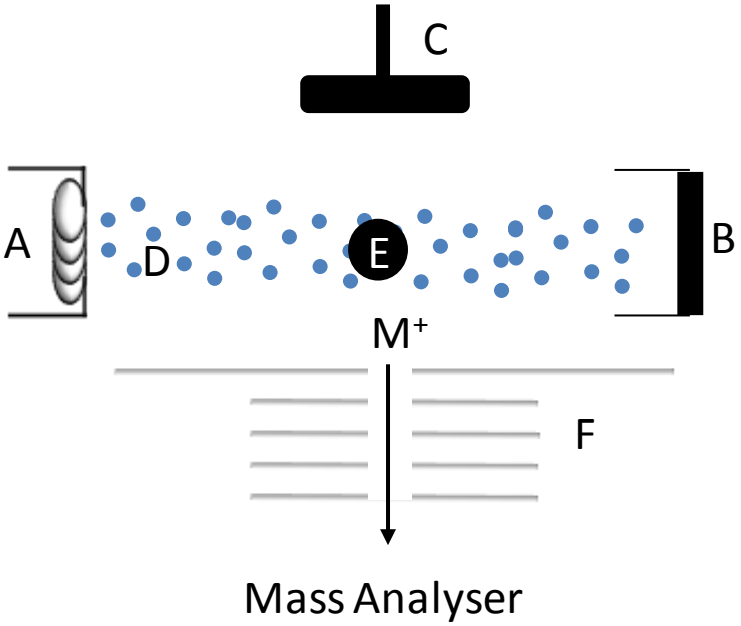


Figure 2.5 Schematic diagram of an electron ionisation source; (A) filament, (B) trap electrode, (C) repeller, (D) electron beam, (E) sample entry on the z-axis, (F) extraction and focussing lenses. The electron beam is directed in the x-plane from the filament towards the trap where analyte molecules are ionised (M^+) and directed towards and focused into the mass analyser by the repeller and lenses respectively.

2.5.1.2. Quadrupole mass analyser

The mass analyser region of the mass spectrometer separates ions according to their *m/z* ratio. Ions generated in the ionisation source are accelerated into the analyser chamber by applying potential to a series of electrostatic lenses that focus and direct the ion beam. There are a number of mass analysers available that can be divided into two categories, those that allow simultaneous transmission of all ions and those that scan a selected *m/z* range. Scanning mass analysers transmit ions with a selected *m/z* value; a range of m/z values may be selected and transmitted sequentially within each scan [126].

In these studies a scanning mass analyser known as a linear quadrupole was employed. A linear quadrupole utilises oscillating electrical fields in which ions have either a stable, or an unstable, trajectory depending on their *m/z* ratio. Wolfgang Paul showed that in a linear quadrupole these fields could be manipulated to allow selective transmission of an ion with a specific *m/z* ratio from one end of the quadrupole to the other and hence be detected. Ions of other *m/z* ratios would have an unstable trajectory under these conditions and be lost before reaching the detector [145]. A schematic of a linear quadrupole is shown in Figure 2.6.

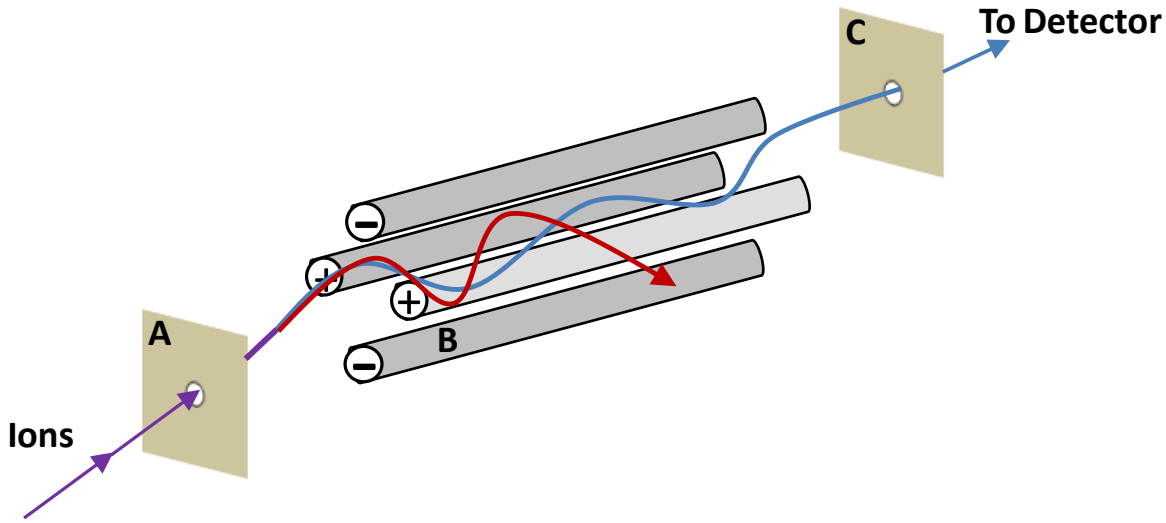


Figure 2.6 Schematic representation of a linear quadrupole. Ions enter the source slit (A) and are directed into the quadrupole, ions of a specific m/z ratio will have a stable trajectory and reach the detector (blue trace) via the exit slit (C) all other m/z values will be unstable and the ions will discharge themselves on one of the rods (B) (red trace).

The quadrupole itself comprises four rods which are electrically connected in opposing pairs, superimposed RF and DC voltages are applied to the rods. A positive ion entering the space between the rods will be drawn towards a negative rod; if the potential on the rod switches polarity before the ion has discharged itself it will change direction. In this way a quadrupole acts as a true mass filter and *m/z* values are detected on a mass selected stability basis [126].

2.5.1.3. Detectors

Several types of detector are used in mass spectrometry to convert the separated ions into an electric current that is proportional to their abundance; ultimately presented to the analyst as a mass spectrum. In these studies an electron multiplier was employed. This converts incident positive or negative ions into electrons which are amplified by subsequent collisions in a cascade effect; producing a current [126].

Key considerations

While mass spectrometry offers many advantages when profiling unknowns there are some key parameters that need to be considered for its successful application to complex matrices. For metabolomic studies mass spectrometers are generally operated in either one of two modes; scanning or selected ion monitoring. If the study is targeting a series of compounds with known common fragment ions the mass spectrometer can be programmed to monitor only select *m/z* values this allows the analyser to switch rapidly between a small number of masses, significantly improving signal – to – noise ratios.

For completely unknown samples, or those where a wide range of compounds are likely to be of interest, operating the mass spectrometer in scanning mode will yield the most information. When scanning, complete spectra are rapidly measured between an upper and a lower *m/z* value defined by the user and the time allowed for each *m/z* value, known as the scan time, is a key factor affecting both the sensitivity of the detection and the accuracy of the obtained spectra.

Suppose a chromatographic peak was to have a width of 10 s, it stands to reason that at least one complete spectrum (i.e. signals from every *m/z* value in the defined range) must be obtained every 5 seconds to ensure that a complete spectrum lies entirely within the peak. If the defined mass range was m/z 35-350 that means that 300 m/z values must be selected during those 5 seconds and so a scan time of 0.017 s per atomic mass unit (amu) can be used. Increasing the scan time increases the amount of time allowed for ions of a particular *m/z* value to reach the detector, thus increasing the number of ions detected and so the sensitivity. This comes with a sacrifice, longer scan times must mean that there will be fewer scans across a chromatographic peak, reducing the fidelity of the chromatographic peak and heterogeneity of the mass-spectrum. This poses a particular problem for complex samples where chromatographic peaks are unlikely to be fully resolved and deconvolution algorithms (Section 2.6.3) will be required to separate and assign the correct ions to the correct contributing spectrum – the higher the number of scans across a peak the more spectral information is present, leading to more accurate deconvolution. A balance between these two factors will be required in profiling studies to provide sufficient sensitivity whilst maintaining sufficient spectral information for deconvolution and confident identification of co-eluting unknown compounds.

2.6. DATA PREPARATION

The output from a GC-MS system operating in scanning mode as detailed in Section 2.5 is typically a total ion current (TIC) chromatogram. The x-axis of a chromatogram represents retention time (t_R) and the y-axis signal intensity, represented as absolute intensity or relative to the most intense signal. A TIC represents the summed intensity across the *m/z* range on the y-axis with retention time on the x-axis.

Figure 2.7 shows an example TIC chromatogram of a skin VOC sample, immediately obvious is the complexity and the wide dynamic range of detected compounds. Data such as this, with typically 300 to 600 resolved and unresolved peaks and intensities spanning several orders of magnitude presents a significant data processing challenge in profiling studies where every peak, no matter how large or small, may be significant.

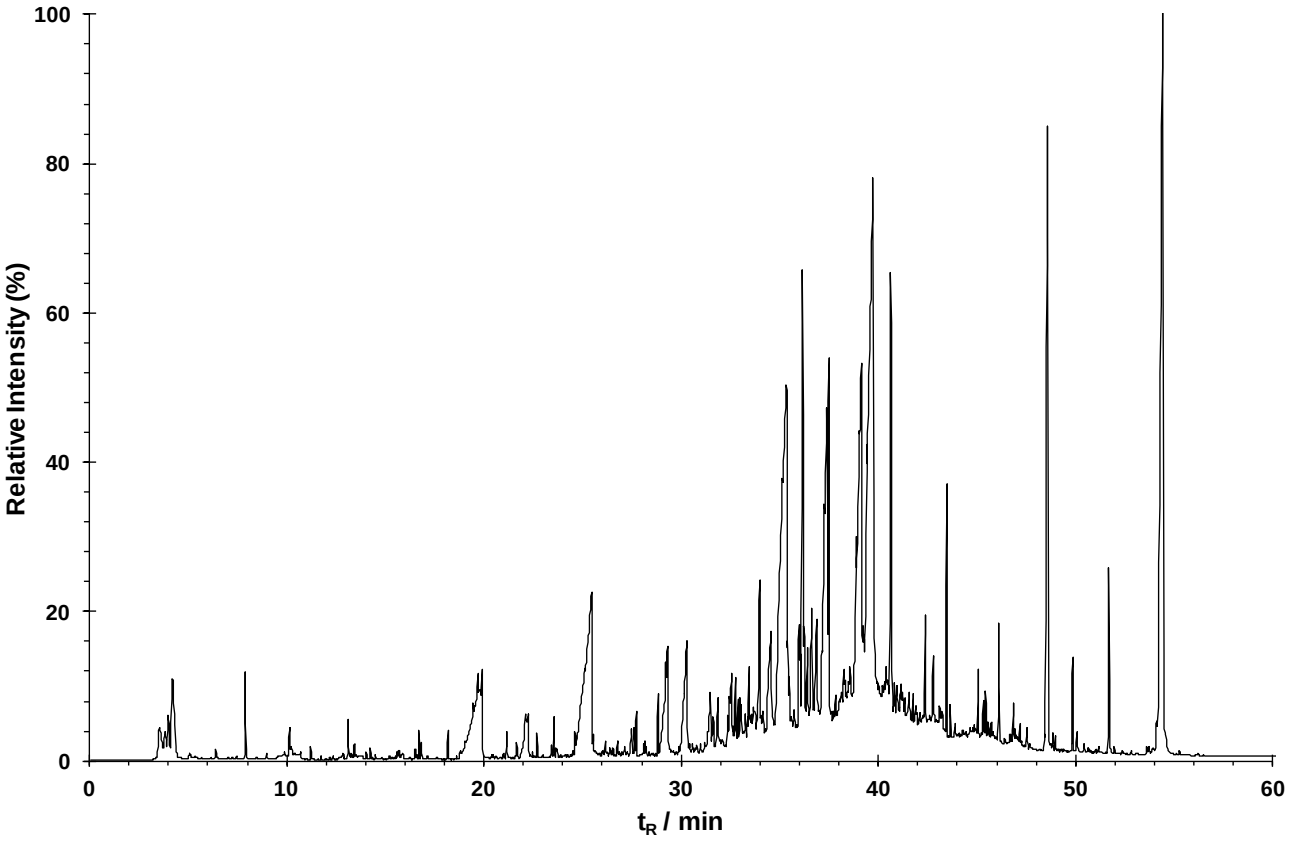


Figure 2.7 An example total ion current chromatogram (TIC) of the VOC profile from a skin patch analysed by TD – GC - MS.

The data handling workflow developed here, summarized in Figure 2.8, is based on that published by Guallar *et. al.* for metabolomic profiling of exhaled breath [70] and has been adapted to accommodate for the added complexity associated with skin profiles.

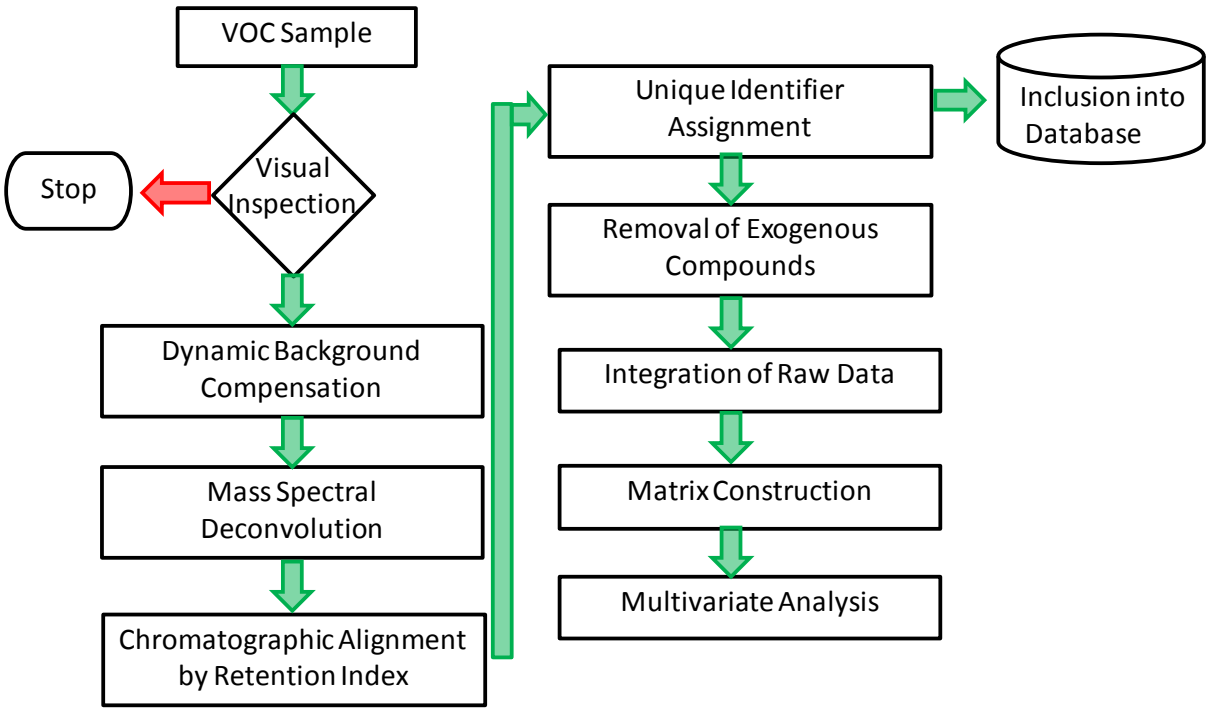


Figure 2.8 A schematic representation of the work flow used to process data from VOC samples. Visual inspection of the data confirms its validity before background interferences are removed by dynamic background compensation and deconvolution provides mass spectral lists. Alignment by retention index compensates for any drift in chromatography and each new compound is assigned a unique identifier based on its retention index and deconvolved spectral list and catalogued. After removal of exogenous compounds the data is incorporated into a matrix for multivariate analysis. This workflow was adapated from that devised by Guallar *et al* reference 70.

2.6.1. Visual inspection

The first step in data processing is the visual inspection of raw data using the instrument's data handling software, in this case Waters MassLynx (V4.1 SCN 779). Whilst regular quality control standards and system blanks verify the performance of the instrument, visual inspection of each chromatogram enables any instrument failures, chromatographic interferences or confounding factors to be rapidly identified and assessed before proceeding with more automated data processing steps. Monitoring known column bleed ions such as *m/z* 207 for polysiloxane columns can give a good indication of instrument response between runs. Spikes due to high water loading or ion suppression due to overloading of volatile analytes towards the beginning of a chromatogram can also be observed by a simple visual inspection of the chromatography which may confound automated deconvolution and integration techniques employed further down the workflow.

2.6.2. Dynamic background compensation

Once a data file has been passed by visual inspection can then be imported into TargetView (ALMSCO International, Llantrisant, UK) for dynamic background compensation (DBC) and mass spectral deconvolution.

DBC algorithms distinguish between ions that contribute to chromatographic peaks and those that can be attributed to noise, suppressing ions from column bleed, oil hills and air/water ingress leaving spectra of much enhanced purity. Signal – to – noise ratios are greatly enhanced for low level compounds after DBC, particularly in a matrix with a wide concentration range [146]. Figure 2.9 shows enhancement from DBC.

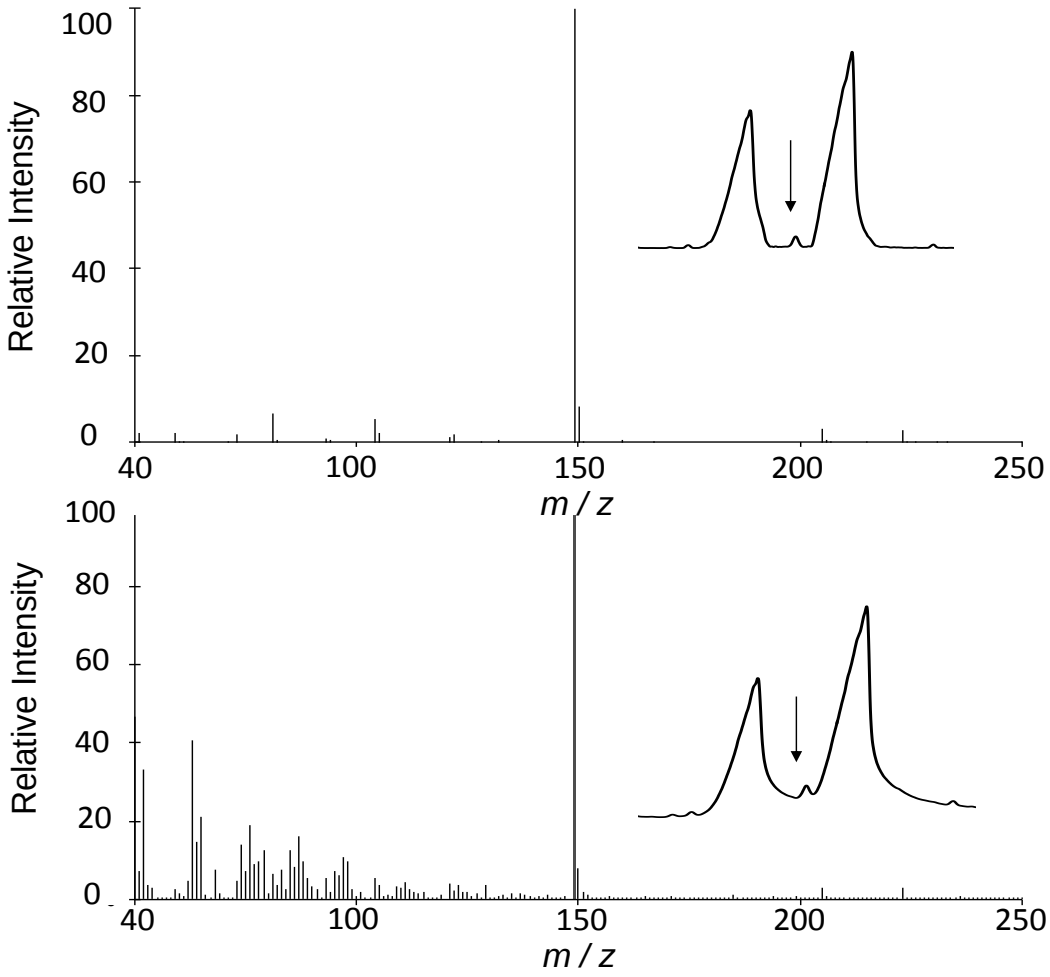


Figure 2.9 Improvement in spectral quality of the mass spectrum of plasticizer dibutyl phthalate, an artifact from the skin patch, by dynamic background compensation. Bottom: The tailing from the preceding chromatographic peak(inset) introduces noise into the mass spectrum without DBC. Top: The influence of the tail from the preceding peak is reduced (inset) and a purer mass spectrum is achieved after DBC. The NIST forward matched for dibutyl phthalate before and after DBC are 589 and 849 respectively.

Important parameters to consider when using DBC algorithms are peak width and noise counts. The noise counts value is an absolute value below which spectral peaks are attributed to noise. More importantly for skin and saliva samples it may be that different peak width values give optimal peak shapes for different classes of compound. Figure 2.10 shows an extracted ion chromatogram for octanoic acid and octanal on a DB-5 column (used throughout this research). The shape of the octanoic acid peak is due to its higher polarity relative to the column stationary phase and is an unfortunate consequence of using one column for a wide range of analytes. This is an important reminder that optimisation of automated data handling techniques is equally as important as optimisation of instrumental parameters. This will be discussed in more detail in Section 3.5.1.

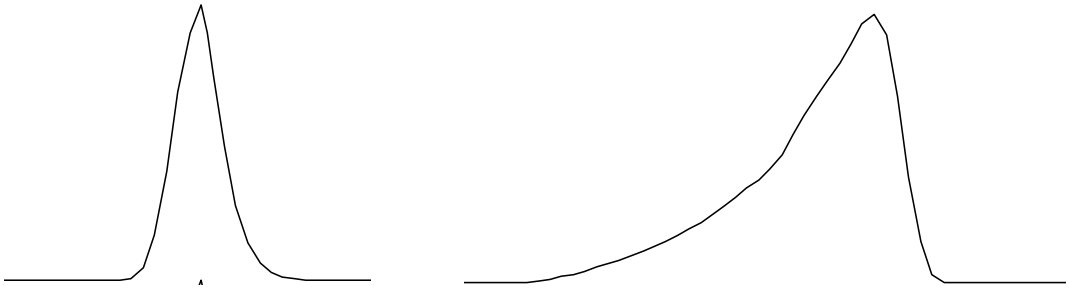


Figure 2.10 Extracted ion chromatograms for octanal (*m/z* 56, left) and octanoic acid (*m/z* 60, right) showing the different peak shapes obtained from VOC profiles using the methods applied in this thesis. Chromatographi peak shape is a key consideration in the development of automated data processing methods.

2.6.3. Mass spectral deconvolution

Ideally all compounds eluting from the GC column would be baseline separated, that is to say that there is no overlap at all between the peaks. For complex samples such as skin and saliva profiles complete separation of every compound is highly unlikely and would be likely to require prohibitively long analysis times or extra instrumentation such as two – dimensional chromatography systems which bring a significant financial and operational burden. To alleviate this, spectra from co-eluting compounds can be separated and attributed to the individual components by a process known as deconvolution. Deconvolution algorithms process the mass spectral data for each scan and assign the ions to the appropriate contributing compound, generating a deconvolved spectrum of only the ions originating from that compound, giving more accurate mass spectra for use as a target against which other samples can be compared or for searching against mass spectral databases [146].

Figure 2.11 shows a representation of mass spectral deconvolution and the resulting 'deconvolved spectral list' for the co-eluting compounds.

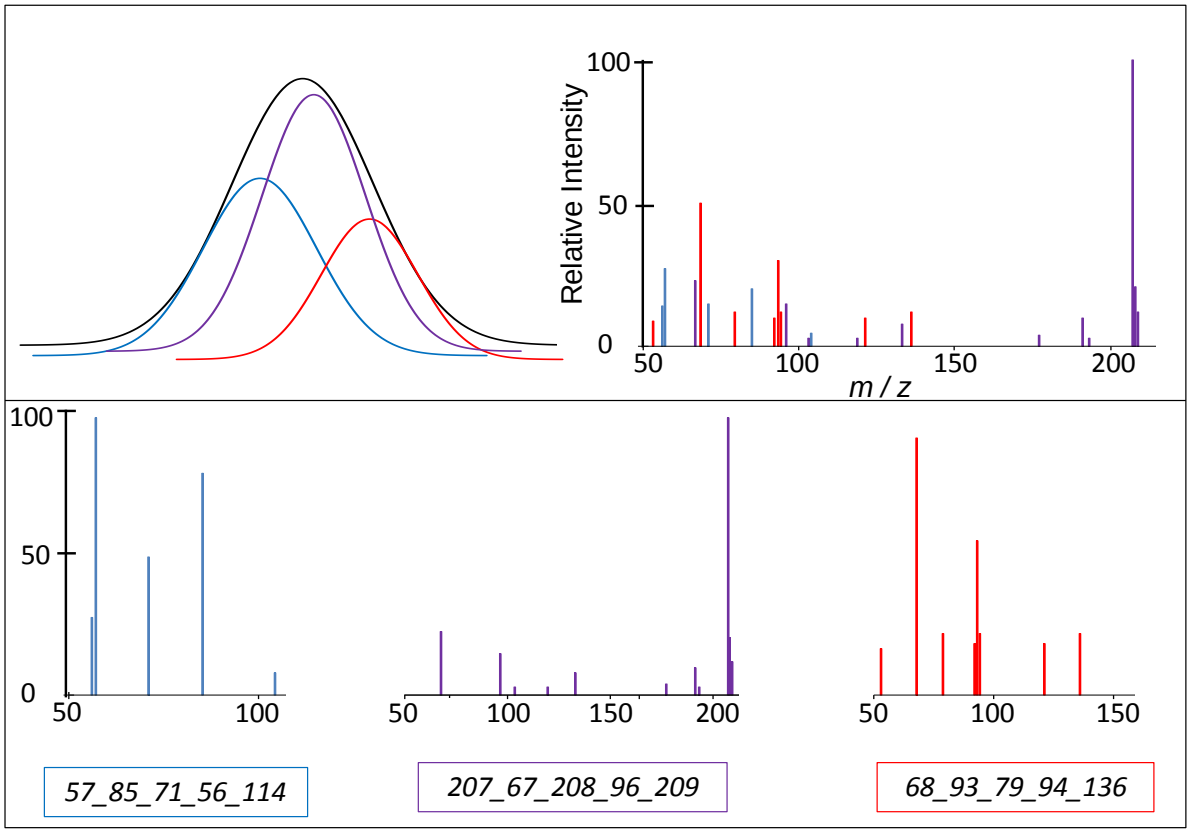


Figure 2.11 **A schematic representation of mass spectral deconvolution showing; top left, 3 components contributing to one chromatographic peak and top right, the resultant composite mass spectrum. Deconvolved mass spectra and spectral lists for each component are shown at the bottom.**

As with DBC the input peak width parameter in TargetView can have a significant effect on the resultant deconvolved spectrum and careful optimisation of this parameter is essential to achieve accurate spectra.

2.6.4. Chromatographic alignment by retention index

Many metabolomic studies require long term stability of analytical instrumentation to enable studies to be performed over an extended time period without the complication of long term sample storage. Deterioration of column stationary phases due to high levels of water and organic acids in biological samples can cause retention times to drift and present a significant problem for these kinds of applications [70]. For many studies employing mass spectrometric detection and monitoring a small number of target compounds this does not present too much of a difficulty,

simply matching the target mass spectrum to a library or widening selected ion monitoring windows may be enough to accommodate for this slippage of retention time. Metabolomic profiling studies however typically screen hundreds of unknown compounds in every chromatogram, many of which have common ions in their mass spectra; to confidently assign a chromatographic peak to the same compound between the first and last samples matching of more than one criteria is required. Many proposals favour the use of an internal standard against which the relative retention time of each component in the mixture is calculated [147]. One considerable disadvantage, however, is that as the retention gap between the analytes and the standard is increased a reduction in accuracy is likely to occur.

The most widely accepted solution to chromatographic alignment is based on a system developed by Kováts in 1958. He showed a homologous series of *n*-paraffin standards eluting in isothermal conditions to have retention times increasing exponentially with the number of carbons in the chain, giving a 'ladder' of standards over the entire chromatographic run. Kováts based his retention index system on the retention time of an analyte relative to the standards eluting either side of it, demonstrated in Figure 2.12 [147].

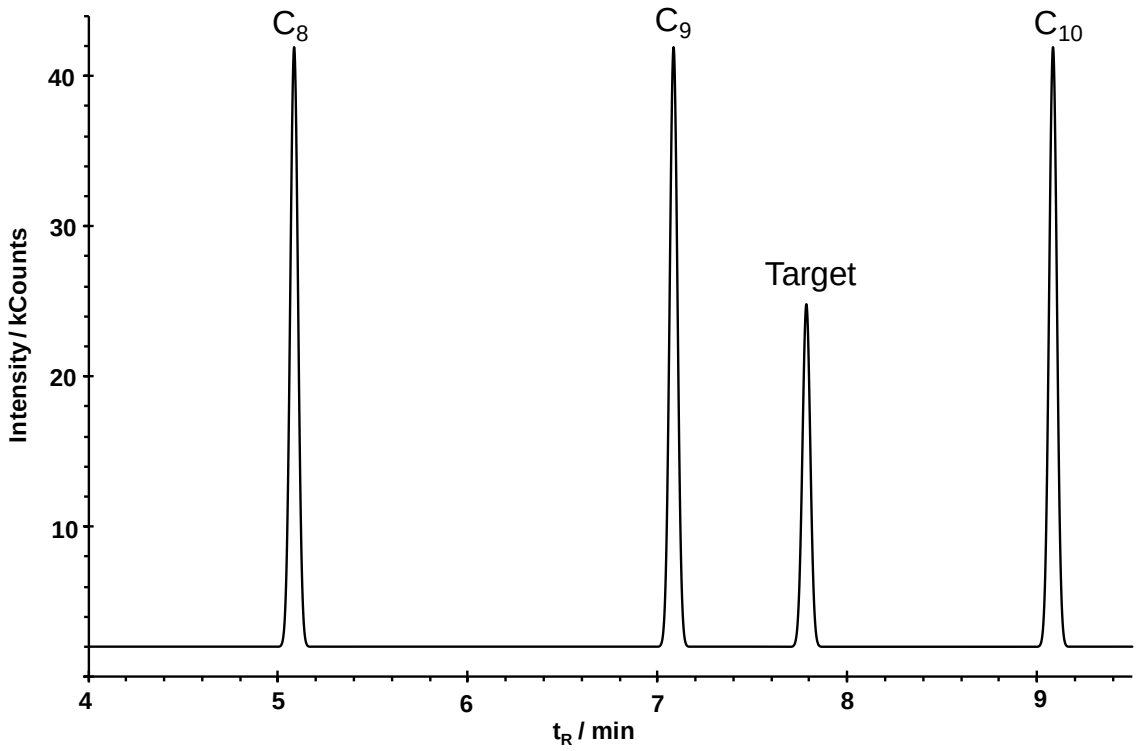


Figure 2.12 Example TIC for a Kováts retention index ladder ladder, C_8 – C_{10} show a homologous standard series used to create the retention index ladder. The target compound's retention index will be calculated based on the standards eluting either side of it; C_9 and C_{10}.

By definition the retention index value of the *n*-paraffin standards is the carbon number multiplied by 100 for any given stationary phase at any given temperature. The target compound identified in Figure 2.12 would be given a retention index relative to the C_9 and C_{10} paraffins using the Kováts Equation:

Equation 2. 6

$$I_s^{st.ph}(T) = 100\left[z + \left(\frac{\log t_s - \log t_z}{\log t_{z+1} - \log t_z}\right)\right]$$

I = Isothermal retention index at temperature (T) on a specified stationary phase (st.ph)
t_s = Adjusted retention time of target compound.
t_z = Adjusted retention time of n – paraffin with a carbon number of z.
$t_{(z+1)}$ = Adjusted retention time of n-paraffin with a carbon number of z + 1.

Any analytical system using the same stationary phase and the same isothermal temperature programme should give comparable values when the Kováts retention index is applied for any compound [147] [148], allowing not only alignment within one study but comparison with other studies, other instruments and even published data. To this end Kováts indices for many compounds at various temperatures on various stationary phases have been reported over the years [149].

The Kováts retention index has been described as the most significant contribution to GC since the discovery of the technique by James and Martin in 1952. Limitations of the method become apparent when temperature programmes are applied to the chromatographic method [148]. Fifty years after its conception the Kováts retention index system is still widely applicable to isothermal and some temperature programmed gas chromatography experiments. The limitations of Kováts indices for temperature programmed methods are discussed in detail elsewhere [148] [150], however provided the system is applied to a fixed chromatographic method using the same stationary phase and method parameters Kováts indices provide an excellent solution to chromatographic alignment within a study.

The application of a retention indexing system based on that of Kováts will be discussed in Section 3.5.3. Following mass spectral deconvolution and retention indexing VOCs from skin and saliva samples can be assigned a unique identifier based on retention index values and the most intense ions from their deconvolved mass spectra.

2.7. MULTIVARIATE ANALYSIS

Multivariate analysis is the application of multivariate statistics, in which more than one statistical variable is simultaneously observed, thus avoiding the loss of any information and generating stable models for predicting underlying trends in latent variables. As GC-MS is capable of generating large data sets and VOC profiles have been demonstrated to be highly complex with hundreds of components in every sample the ability to process and monitor changes across the entire profile is essential when prospecting for markers.

For multivariate analysis data are arranged into a matrix with each sample being denoted an observation and each individual compound (classified by the unique identifier described in Section 2.6) denoted a variable, the matrix structure is shown in Figure 2.13. The matrix is then populated with a response value for each variable in each observation, for GC-MS metabolomics this is usually the area of the chromatographic peak.

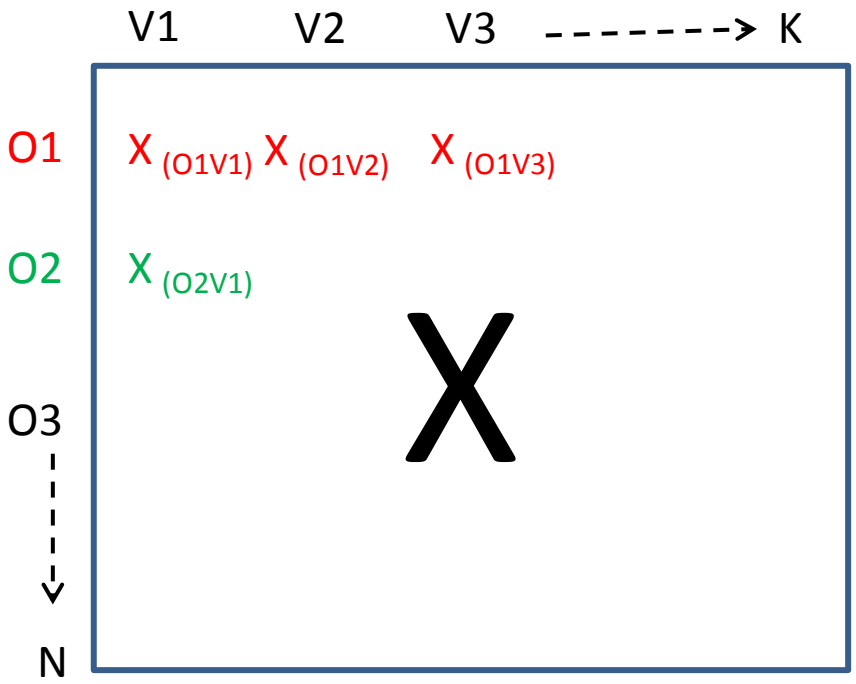


Figure 2.13 Multivariate analysis matrix for the analysis of VOS samples consisting of k variables (columns) and N observations (rows). X represents the response (chromatographic peak area) for each variable in each observation.

There are many multivariate statistical models available for investigating variance within a data set, two of the most commonly used method in metabolomics studies are principle component anlaysis and partial least squares discriminant anlaysis.

2.7.1. Principle component analysis

Principle component analysis (PCA) is a technique for reducing the dimensionality of a data set whilst maintaining the maximum amount of variation possible [151]. Data is transformed into a new set of uncorrelated variables known as principle components (PC) by linear combination of the original variables in such a way that the initial PC accounts for the most variance in the set, the subsequent PCs are calculated successively.

A detailed description of the mathematical basis of PCA can be found elsewhere [152-153], a brief description is included here. A geometric space is constructed with k dimensions, k represents the number of variables so each variable has its own dimension, and each observation is then represented as a point in 'k dimensional space'. Principle components are constructed by transforming the axis of the original plot so that the new vectors lie along the direction of maximum variance. The projection of the second principle component is orthogonal to the first in the direction of the next highest variance and so on.

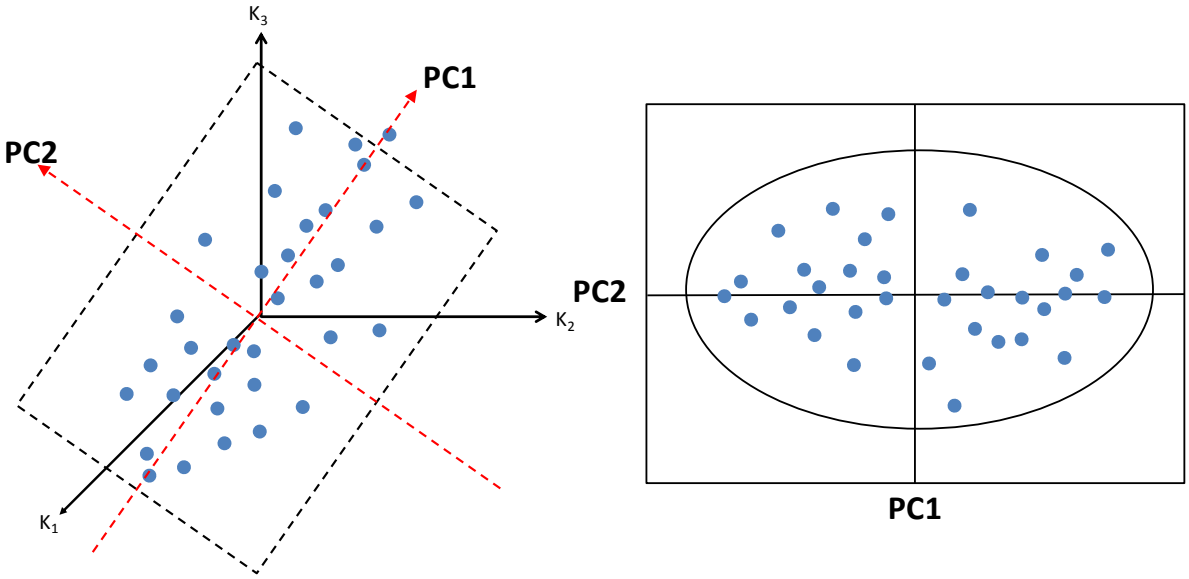


Figure 2.14 Visualisation of principle component analysis (PCA) of a 3-dimensional data set. The left hand plot shows an example of the first two principle components (PC1 & 2) being projected onto a 3-variable data set (K_{1-3}). PC1 explains the highest amount of variance, PC 2 the next highest. The right hand plot shows the data re-plotted on axes according to PC1 and PC2, a scores plot. The distance of each observation along the PC1 and PC2 axes is called a score and is calculated based on their distance from the origin of the original k-dimensional space.

Once the principle components have been calculated the data is re-plotted against new axes based on the new principle components, this is called a scores plot and can be generated for any two PCs from the data set. The scores for each observation are calculated as the distance of the

point (observation) from the origin after projecting the data onto the new principle component and are represented by the position of the observation on the scores plot, Figure 2.14.

The amount of variance in a data set explained by a principle component is known as the eigenvalue and the number of principle components to be calculated is decided by the analyst, the percentage of variance explained by each principle component decreases for each successive component so any real differences between classes are most likely to be observed in the earlier principle components [154].

In metabolomic studies PCA is principally employed to determine differences between classes [153], diseased or healthy for example, and observations tend to be plotted in terms of class to enable a complex data set to be represented in such a way as to observe difference with the naked eye.

2.7.2. Partial least squares discriminant analysis

While PCA is an unsupervised method, that is to say no metadata is input into the model, PLS-DA applies a training set of data, where the class information is known, to fit the remaining unknowns to the correct class, i.e. it is a supervised method.

PLS-DA models use prior knowledge to find the maximum separation between the defined classes by computing a linear discriminant function, Y, as a combination of the original variables $K_{1.....N}$. Validation of such models is often performed as part of model generation in programmes such as SIMCA-P where a 'leave one out' validation method is employed [155]. In this method the data is divided into a training set, comprising 70% of observations, and a test set, the remaining 30%. The model, Y, is then computed based on the training set and the test set used to determine the accuracy of classification this is an iterative process that continues until each observation has been included in the test set, or 'left out', once and the model has determined the optimum result.

"Perturbation of a biological system causes myriad changes, only some are directly related to the cause" [155].The use of supervised statistical models such as these can be controversial as it can be seen as forcing the model to fit the data and it is important when using them to remember that statistical significance does not always relate to biological significance and it is important to remember to relate findings to the original raw data after such complex processing.

As demonstrated by Turner *et al* a combination of multivariate models can often provide the optimum balance between the discrimating power of supervised models and the confidence associated with unsupervised [62]. For highly complex matrices such as human skin, saliva and breath there are likely to be a high number of factors causing variation within a data set other than the one under study. For example it is difficult to recruit a set of volunteer participants for a panel

study that all have the same body mass index, diet, age and gender and these factors will all contribute to the variance within the data set. Applying supervised approaches such as PLS-DA the analyst is able to ask the model to find any variables that may cause variation due to a known intervention or disease under study, the variables predicted by such a model can then be extracted and used in an unsupervised model such as PCA. In that way variation due to extraneous factors can be screened out and unsupervised PCA then used to determine whether these variables do correlate to variation between the classes under study. The utility of this approach in pilot metabolomic studies will be demonstrated in subsequent Chapters.

2.8. CONCLUSIONS

Consideration of the complexity of the challenges associated with designing and implementing non-invasive diagnostic sampling and analysis techniques emphasises the need for careful optimisation and control of a wide range of factors. For example the thickness of sampling phase and sampling time, through to the balance between good chromatographic separation and analysis time and high sensitivity with accurate mass spectra ultimately leading to data handling techniques such as background compensation, deconvolution and statistical modelling. All have the potential to introduce bias to a study and so confound the research.

The exploration and optimisation of these factors for global metabolomic profiling of human skin and saliva (Chapter 3) is likely to involve a series of considered compromises in an attempt to gain the most representative snapshot of the metabolic profile of an individual at a moment in time in a simple, robust, reproducible and most importantly non-invasive, safe and comfortable manner.

3 THE DEVELOPMENT OF A GLOBAL VOC PROFILING WORKFLOW: CHARACTERISATION AND OPTIMISATION OF SAMPLERS AND ANALYTICAL METHODOLOGY

The previous Chapter discussed the overall workflow for a metabolomic study and outlined key factors and parameters that must be considered for each stage. In this Chapter the characterisation of non-invasive VOC samplers is presented alongside optimisation of instrumental methods and data processing strategies for global VOC metabolite profiling of human skin and saliva.

3.1. ETHICS AND PARTICIPANT PREPARATION

3.1.1. Application for ethical approval

The studies were conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki. Approval from the Ethical Advisory Committee of Loughborough University was obtained for the generic sampling protocols for VOC sampling from skin and saliva (reference number G10-P7 and G10-P23) and for each specific study.

At the outset a protocol outlining the details of the study was prepared to include a brief rationale, a hypothesis and description of prospective participants including inclusion and exclusion criteria. The protocol then described the preparation and sterilisation of the sampling device, the mode of sampling and any potential risks, the protocols in place for transport, storage and destruction of human biological samples and finally the storage, protection and distribution of data relating to human participants. Once complete the protocol was appended to a generic protocol approval form for human biological investigations and submitted to the ethical advisory committee along with example participant information sheets, consent forms and health screen questionnaires. An example ethical approval application bundle is included in Appendix 1.

The ethical advisory committee reviewed each protocol and any questions or requested amendments were passed back to the researchers to be addressed. The researchers then responded in writing to the Chairman of the committee. The applications submitted for studies in this thesis required only minor points of clarification so the Chairman was able to approve these without convening the full committee and ethical approval was obtained within ca. 90 days before which no participant recruitment could commence.

Volunteer participants were recruited from Loughborough University Staff and Students and consisted of healthy adults aged 18-35. Participants were given a brief description regarding the nature, purpose and possible risks of the study together with the participant information sheet and were encouraged to ask questions before an enforced 24 hour cooling-off period, only after which could they decide whether to participate in the study at which point they gave written informed consent. A chaperone of the same gender as the participant was present during sample collection and access was restricted to only those researchers and participants involved in the sampling process.

3.1.2. Participant preparation

To limit the potential confounding factors associated with sampling from humans, participants were requested not to eat, drink anything other than unflavoured water, brush their teeth or use any personal care products on the morning of any study visit. As a result of the fasting requirement all study visits were scheduled to commence before 11 am. In the 24 hours prior to the study visit participants were also requested to refrain from drinking alcohol or exercising. Any other more study specific requirements will be discussed in the relevant Chapter.

3.2. SAMPLING OF VOLATILE ORGANIC COMPOUNDS FROM HUMAN SKIN: THE SKIN PATCH

In Chapters 1 and 2 the use of a polydimethylsiloxane (PDMS) membrane to sample VOCs from the skin surface was discussed; based on the work of Riazanskaia *et. al,* this method is known as the Skin Patch [111]. The Skin Patch, shown in Figure 3.1, is a PDMS coupon 5 mm x 15 mm x 0.45 mm that conforms to the contours of the human body, held in place by a cotton-wool pad, to absorb VOCs from the skin lipid layer. Detailed characterisation studies of the Skin Patch have been described elsewhere, and are supported by *in-vivo* applications such as profiling chronic wounds and bacterial contamination of meat [34] [111] [156]. This work focuses on the factors relating to the application of the Skin Patch to metabolomic studies of VOC profiles of human skin.

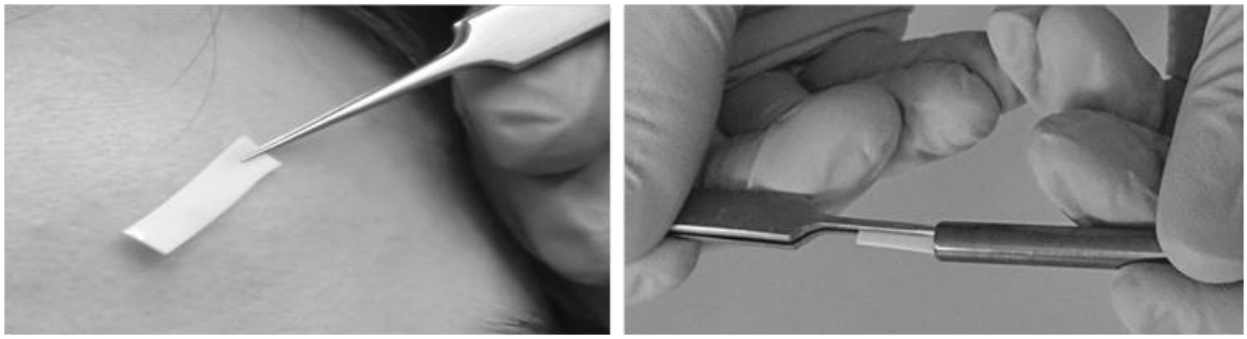


Figure 3.1 Left: PDMS Skin Patch being applied to a participants forehead, and right: Skin Patch being transferred to an empty stainless steel thermal desorption tube for storage and analysis.

In-vitro and *in-vivo* reproducibility studies [111] with the Skin Patch, by Riazanskaia *et. al*, showed recovery and precision to correlate with volatility; higher molecular weight, higher boiling point compounds exhibited higher recoveries and lower relative standard deviation in both *in-vitro* and *in-vivo* data. This was attributed to more volatile species being more sensitive to small variations in sampling procedures (faster permeation, diffusion and desorption kinetics), yet the authors were able to conclude that the Skin Patch offers a robust, predictable and reliable method for sampling VOCs from human skin [111]. It was also observed that the amount of VOCs recovered by the Skin Patch increased with reducing volatility. Even so, compounds across the volatility range exhibited a linear response with regression coefficients in the range 0.95 to 0.998 for sampled mass versus response. The conclusion was that the amount of VOCs recovered from a Skin Patch sample reliably reflects changes in the concentration of analytes on the skin. The data presented by Riazanskaia, and the independent follow up studies [34] [156], demonstrated the utility of the Skin Patch approach for sampling VOCs from skin and this approach was adopted for this research. The remainder of this section describes the Skin Patch sampling and analysis protocols and their optimisation for global profiling studies.

3.2.1. Sampling time

Riazanskaia and co-workers investigated the effect of *in-vivo* sampling time on the recovery of 5 randomly selected compounds across the human skin VOC profile. The collection of most compounds appeared to rapidly increase in a non-linear fashion over 5 to 12 min of sampling [111]. After this there was a general trend of recovery increasing linearly with sampling time. The exception to this was the most volatile compound studied, trimethylcarbazole, which rapidly accumulated for the first 5 min of sampling, and any further extension of sampling time resulted in a reduction of the recovery of the compound. This molecule was extracted from the skin, and then

lost to the surroundings, faster than it was being excreted, and the conclusion was that sampling time should be chosen to match the properties of the compounds of interest; higher volatility species are likely to require significantly shorter sampling times than the lower volatility counterparts [111].

The observations in the studies characterising the Skin Patch all link analyte recovery to volatility and highlight the challenges associated with applying one sampling method to a global profiling approach. Guided by the data presented by Riazanskaia *et. al.,* and evidence from unpublished research a sampling time of 30 min was chosen; giving a compromise between increased sensitivity for all but the most volatile, and retention of the lightest compounds.

3.2.2. Conditioning

The published methodology for preparing Skin Patches prior to analysis was tested and found to be insufficient for the current studies. 'Blank' profiles obtained after following the recommended wash with 5 % aqueous Decon 90 solution and 15 hr conditioning under vacuum at 180 ºC contained levels of VOCs up to 50 ng per compound per Skin Patch, whilst some of these were siloxane compounds from the PDMS the majority were polar species such as acids and alcohols. The methodology was improved by an additional step in which the Skin Patches were subject to a methanol wash in an ultrasonic bath which was performed in triplicate with clean methanol in each repeat. This reduced the levels of non-siloxane VOCs in blanks to below detectable levels; less than 10 pg per Skin Patch, calculated as n-decane equivalents.

The VOC contamination from the cotton-wool pads used to hold the Skin Patch against the skin surface was assessed by sampling a cotton-wool pad with a Skin Patch for 30 min. The resultant chromatogram was found to contain many more VOCs than anticipated and so a conditioning procedure was also developed for these in which cotton pads were conditioned under vacuum at 70 ºC for 4 hr individually wrapped in aluminium foil. The cotton pads were then stored and transported in the tin foil wrapping until use, an example blank chromatogram from a cotton pad pre- and post-conditioning is shown in Figure 3.2.

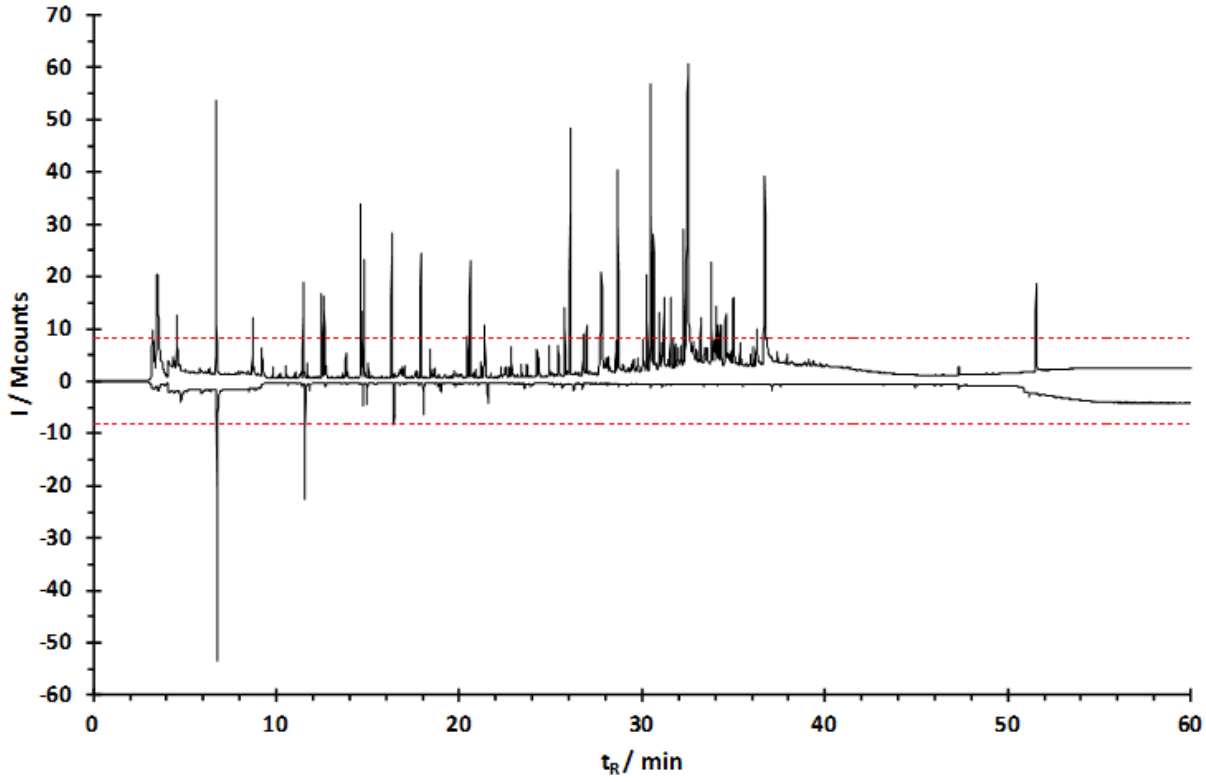


Figure 3.2 TIC of samples taken from a cotton pad used to hold the skin patches on the skin surface. The top trace shows the chromatogram from an unconditioned cotton pad and inverted is the chromatogram from a cotton pad that has been conditioned under vacuum at 70 °C for 4 hr. The red dashed line shows marks the response in M counts to 1 ng in n-decane equivalents.

It is important to note that although conditioned Skin Patches gave reproducible qualitative profiles, the levels of siloxane artefacts observed in blanks was variable, and so while it was possible to exploit the retention time of these compounds within the resultant chromatograms (see Section 3.5.3.), it was not possible to use them as internal standards.

3.2.3. Storage

The published methodology for the Skin Patch reported significant variations in levels of recovered VOCs from samples stored longer than 24 hr at 4°C. The increases observed could not be accounted for by any variations in instrument performance, and were attributed to bacterial activity. Bacteria transferred to the Skin Patch from the lipid layer were thought to be able to survive during storage at 4°C and continue to metabolise on the Skin Patch.

The analysis time of a Skin Patch is in the order of 70 min and in order to use this methodology in larger scale metabolomic studies it is essential that samples can be stabilised for transport from the field or clinic and storage until analysis. With large numbers of samples the time to analysis

could be several days to some weeks. Three options were considered for extending storage time: reducing the storage temperature; sterilisation of the Skin Patch post-sampling with ultra-violet light; or thermal desorption and re-trapping of VOCs onto more stable sorbent systems thereby sterilising the sample with high temperatures. The simplest and least expensive of these was to use lower storage temperatures and storage at -20ºC and -80ºC was studied as a design pilot for the metabolomic studies. A central composite design was used to determine storage times for investigation as 1 (24), 4 (96), 11(264), 18 (432) and 21 (504) days (hours). Five replicate samples were taken for each storage time. In order to simultaneously collect sufficient samples for 5 replicates at each time point a large area of homogenous skin was required. 30 samples were taken from the upper back of each participant for each storage temperature. 6 sampling locations were defined on the participants back and the samples taken from each were randomised to ensure that no bias occurred and each storage time contained samples from randomised areas of the back.

VOCs were selected at random from within defined 10 min periods of the chromatography to provide a representative volatility range and analysis of variance (ANOVA) performed on integrated peaks areas with the null hypothesis that storage conditions have no effect on the VOC profile. After 4 days storage at -20ºC one compound caused the null hypothesis to be rejected, this was 6-methyl-5-hept-2-one which has previously been noted to increase up to 300% on storage at 4ºC [111]. After 11 days storage at -20ºC other VOCs were also exhibiting increased responses causing the null hypothesis to be rejected and at this point the -20ºC storage study was halted and this storage condition was deemed insufficient for the storage of skin VOC samples. It is interesting to note that while several increased upon storage, none of the compounds monitored showed a significant decrease upon storage that would be expected to accompany an increase elsewhere in the profile, indicating that these compounds may be the product of bacterial metabolism of compounds too heavy to be recovered from the Skin Patch and analysed by the method employed here. After 21 days storage at -80ºC none of the monitored compounds showed any statistically significant change and this was considered an adequate storage condition for skin VOC samples in these pilot metabolomic studies. Figure 3.3 shows the effect of storage duration on 6-methyl-5-hepten-2-one from Skin Patch samples stored at -20ºC and -80ºC.

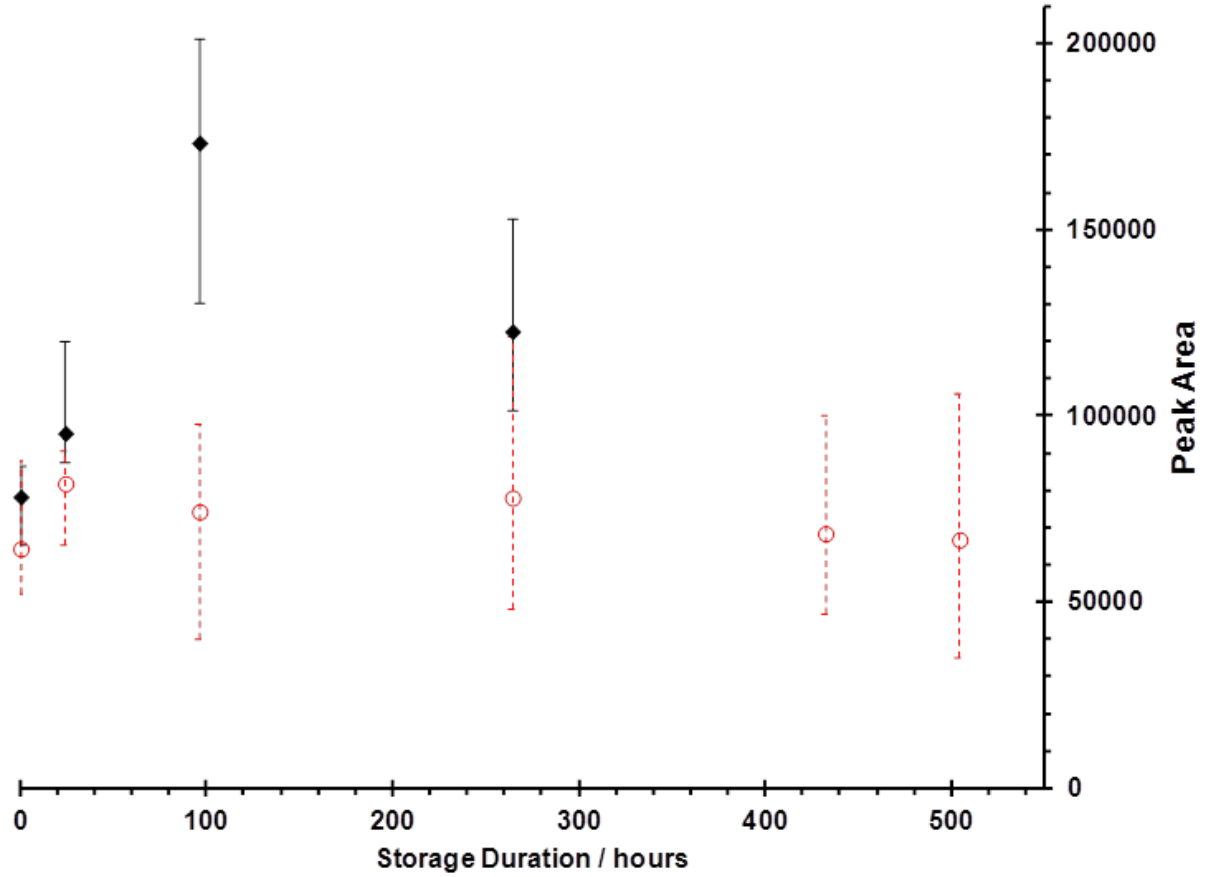


Figure 3.3 The effect of storage on 6-methyl-5-hepten-2-one in human skin VOC profiles. Each data point represents the average peak area and the positive and negative error bars the maximum and minimum area obtained for each point respectively. The black trace shows data for -20ºC storage and the red -80ºC. The increase in 6-methyl-5-hept-2-one levels after 96 hours (4 days) is evident when stored at -20ºC while at -80ºC there is no statistically significant change over 504 hours storage.

It is worth noting that the first attempt at this storage-study was unsuccessful because the seals on the caps of the thermal desorption tubes failed. This was judged to be the result of differences in the thermal expansivity of material used (stainless steel tubes, PTFE ferrules and brass caps), causing the caps to become loose. In this study the sample tubes were placed at low temperature for 30 min and the caps then re-tightened before long-term storage of samples.

The loss of sample integrity at -20ºC after 11 days suggests that cooling may be insufficient to halt sample degradation for long term storage and other strategies might need to be evaluated for longer term storage of skin samples for larger scale studies, or a larger extended sample storage study undertaken.

3.2.4. Summary of non-invasive skin sampling

This research used a simple, robust, easily portable, non-invasive sampling methodology to collect VOC profiles from human skin. A review of published methods has shown VOC profiles obtained using the Skin Patch to be reproducible and representative of concentrations at the skin surface [111].

The importance of sampling time has been discussed and 30 min was selected to give the optimum global profile recovery across the wide volatility range expected from human skin samples.

Enhancements in the quality control of blank samplers ensured conditioning protocols for both the Skin Patch and the cotton-wool pad covering consistently produced blank VOC profiles with contaminant levels sufficiently low to allow the method to be applied to the global profiling of unknown samples.

Finally investigations into longer term storage solutions confirmed that maintaining sample integrity with storage is a critical factor in the design of metabolomic studies. VOC skin profiles were found to be stable for up to 21 days when stored at -80ºC. The ability to confidently store skin VOC samples for 21 days is an important finding for transferring this methodology to larger scale studies.

The size and shape of the Skin Patch lend it the versatility to be applied to all but the most delicate structures of the skin surface and careful consideration of sampling site was, and will be, necessary when designing studies. The sampling sites chosen in this thesis were study specific and will be discussed in detail in the relevant sections. The protocols described in this section for preparing, sampling and storing skin VOC profiles from human participants were used for global VOC profiling in pilot metabolomic studies to assess the utility of skin sampling alongside existing non-invasive protocols.

3.3. SAMPLING VOCs FROM HUMAN SALIVA

In line with skin VOC sampling methodologies a polydimethylsiloxane (PDMS) based sampling approach was employed for saliva sampling, this strategy for selectively sampling VOCs from human saliva has been successfully applied in the form of solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE) of expressed saliva samples [50] [133] [157].

The difficulties associated with collecting a representative saliva sample, briefly comprising techniques for inducing salivation, the relative increase in collected bacteria when saliva is collected by spitting versus drooling and the potential losses of VOCs during sampling were

reviewed Section 1.4 and highlighted the potential confounding factors that must be controlled, despite the apparently simple act of collecting an individual's saliva. When sample handling and storage techniques are also examined it becomes apparent that a wide range of factors affect the VOC profile ultimately obtained from a saliva sample, and that the recovery and analysis of many VOCs may be determined as much by the analytical methodology as by metabolism of the individual [47].

In this study PDMS samplers were applied to *in-vivo* sampling of human saliva directly from the mouth, removing the pre-treatment, collection and handling complications associated with collecting expressed saliva.

3.3.1. Designing a saliva sampler

Initially sampling directly from the oral cavity was achieved using a PDMS Skin Patch secured using lockable haemostats with blunt ends, the participant was able to place the PDMS patch either under the tongue or inside the cheek whilst holding the handles of the haemostats outside the mouth. While this approach was effective participants found both the corners of the coupon and the hemostats to be uncomfortable and difficult to bear for more than a minute or so. While it would be possible to modify the shape, size and thickness of the PDMS coupon to improve comfort and maintain sampling efficiency, the safety and comfort of the participant must be the highest priority in designing an *in-vivo* sampler. The design of a reproducible, inert and safe method for placing and controlling the position of the PDMS coupon in the mouth presented a significant challenge and several configurations were attempted including several cage type approaches, wire leashes and extending the length of the coupon to act as a handle. None of these approaches fulfilled all the stated requirements and the configuration of the PDMS sampler itself was addressed.

Other PDMS sampling configurations were reviewed including SPME, SBSE and SPE-td. A saliva sampler was designed in collaboration with Unilever PLC and machined by Peter Carroll of Unilever. The saliva sampler consisted of a solid phase extraction (SPE) cartridge (Cat no: C-SPTD5-6MM Markes International Ltd), a hollow titanium rod coated with PDMS, which was mounted onto a bespoke holder for sampling. Figure 3.4 shows a computer-aided design (CAD) drawing of the component parts and the assembled sampler, this has been reproduced from the original drawing with Peter's permission, the original may be found in Appendix 2. Table 3.1 summarises the design parameters for the sampler. Sampling was effected by placing the PDMS

portion of the sampler into the oral cavity, underneath the tongue, in an analogous manner to an oral thermometer, as shown in Figure 3.5.

Table 3.1 Summary of the design parameters for the saliva sampler

Parameter	Dimension
Cartridge	
Support	Hollow Titanium Rod
Sorbent	Polydimethylsiloxane (PDMS)
Length	6 mm
PDMS Film Thickness	500 µm
Holder	
Material	Stainless Steel (grade SST-316)
Total Length	101.19 mm
Handle Diameter	5.93 mm
Cartridge Mount Diameter	1.25 mm
Cartridge Mount Head Diameter	3.96 mm

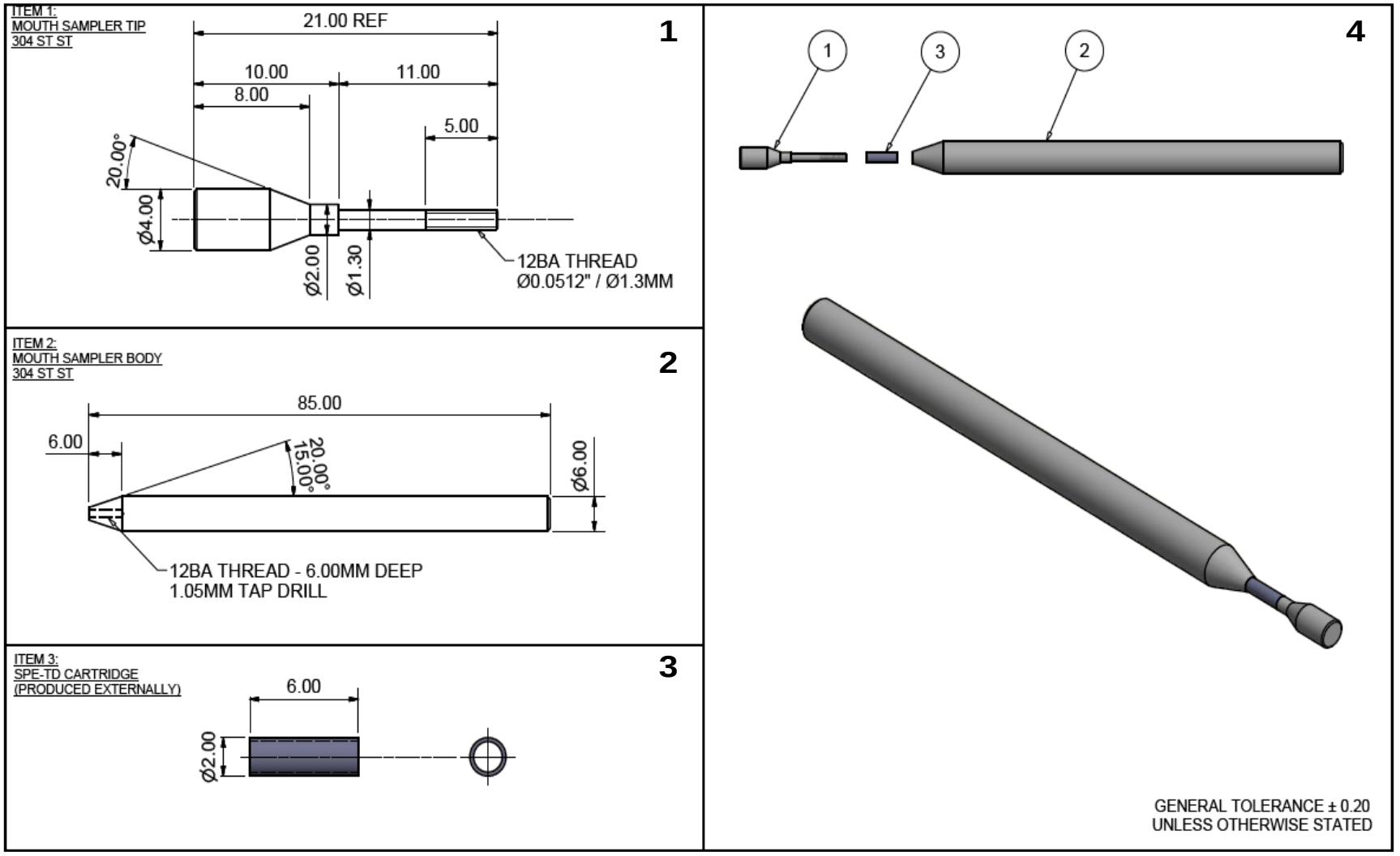


Figure 3.4 Computer aided design (CAD) drawing of the saliva sampler and component parts, all dimensions are in mm unless otherwise stated. Panel 1: Drawing of the sampler head and spindle, 2: drawing of the sampler handle into which the head is screwed, 3: the SPE-TD cartridge, a hollow titanium cylinder coated with PDMS. Panel 4 shows an exploded diagram of the 3 sampler components, the spindle of part 1 passes through the hollow centre of the SPE-TD cartridge and screws into the handle (part 2). The assembled sampler is shown in panel 4, the SPE-TD cartridge is secured between the sampler head and handle and may be safely and comfortably placed into the oral cavity to effect sampling.

Prior to use the cartridges were placed into empty thermal desorption tubes and conditioned with 50 cm^3 min^{-1} of inert carrier gas at 190 ºC for 60 min. Once conditioned, the samplers were thermally desorbed and analysed to ensure that they were free from unacceptable levels of VOC contamination; Figure 3.6 shows an example blank chromatogram compared with a saliva VOC profile. Immediately prior to sampling the saliva sampler was assembled, with care taken not to contaminate the solid-phase extraction cartridges by handling with stainless steel tweezers. The sampling procedure commenced with the participant drinking 50 cm^3 of water to refresh their mouth, 1 min later the participant placed the saliva sampler into the sublingual region of the mouth where they could use the handle to stabilise and reposition the saliva sampler if needed, Figure 3.5. At the end of sampling the participant removed the saliva sampler by drawing it through their closed lips to remove any excess liquid from the surface. The researcher immediately unscrewed the spindle and placed the solid-phase extraction cartridge into a clean, empty glass thermal desorption tube that was then sealed and stored in an airtight container at 4 ºC until analysis.

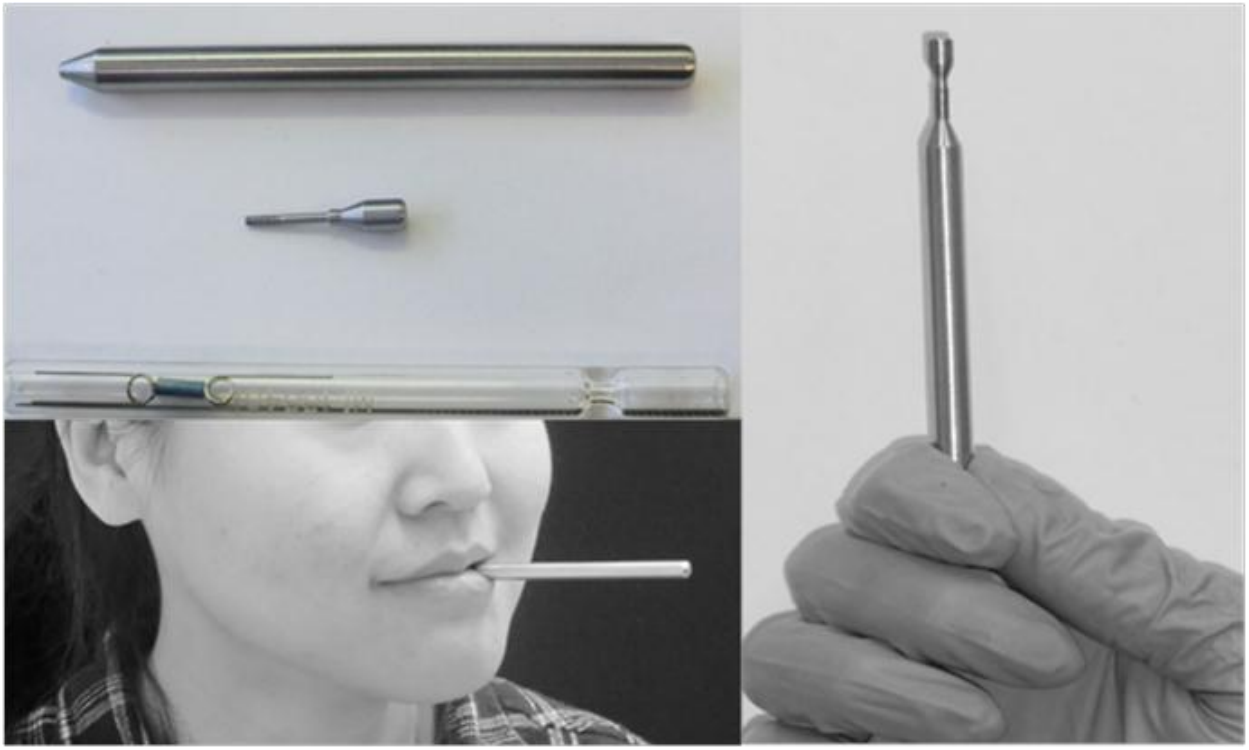


Figure 3.5 Photographs of the saliva sampler. Clockwise from top left. A titanium cylinder coated with polydimethylsiloxane is held in place by two springs inside a glass thermal desorption tube. Once conditioned the cylinder was removed and placed onto the threaded stainless steel spindle that was then screwed into the holder, (Right). The assembled sampler could then be placed comfortably into the mouth of a participant where a VOC sample was collected *in-vivo* (Bottom left).

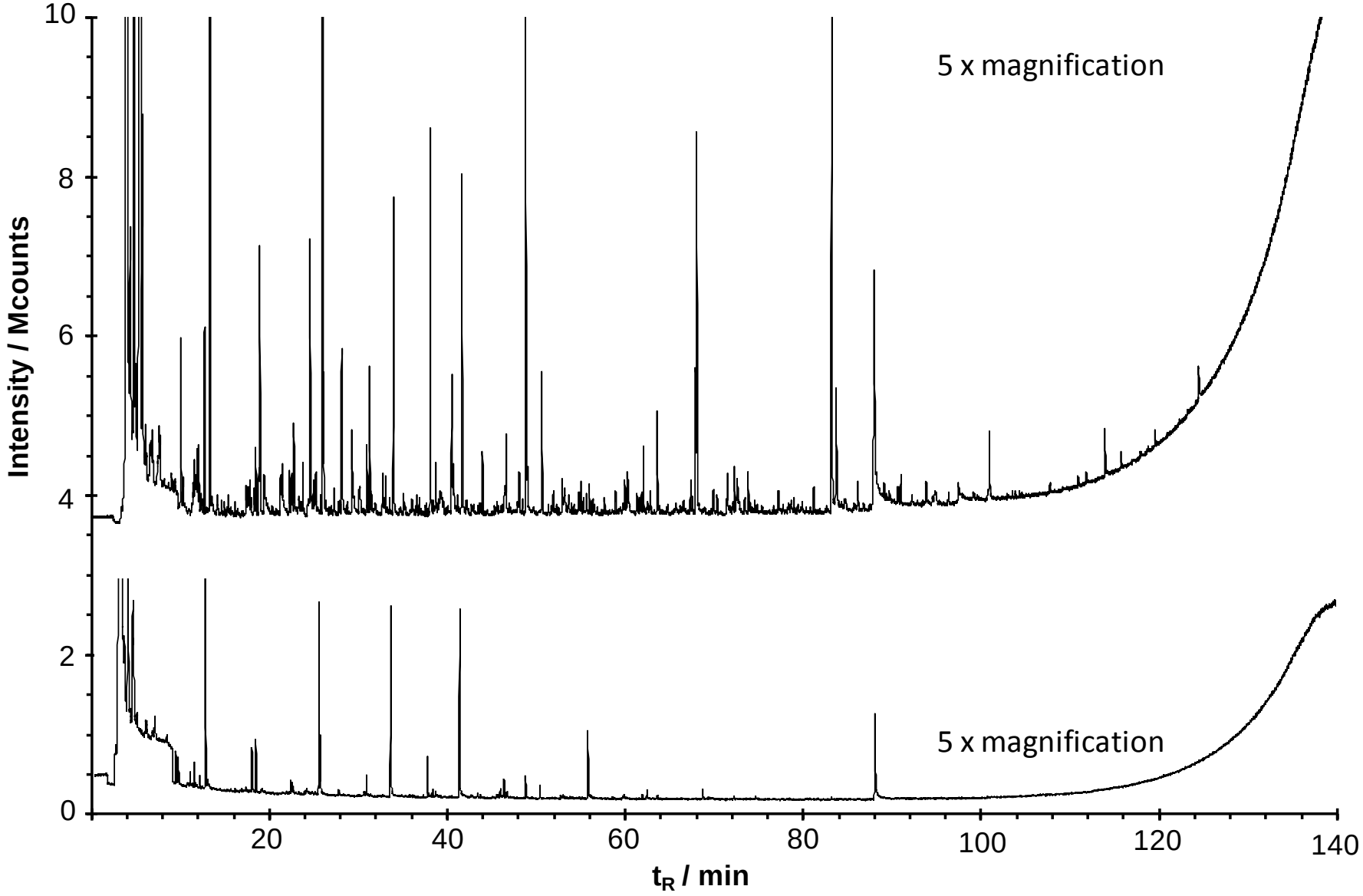


Figure 3.6 Top: an example TIC chromatogram of a VOC saliva profile obtained from *in-vivo* sampling with the SPE cartridge sampler. Bottom: the corresponding response from a sampler blank. Traces are magnified 5 fold with a 3Mcounts cut off for the blank and 10 for the saliva trace, the saliva trace is offset at 3.5Mcounts.

3.3.2. Saliva sampler characterization

The suitability of the saliva sampler for global profiling was assessed in a series of characterisation experiments to determine key sampling considerations and produce analytical figures of merit [47].

3.3.2.1. Reproducibility

The reproducibility of the technique was assessed both *in-vitro* and *in-vivo*.

In-vitro reproducibility was determined using an aqueous 1 ppm (v/v) standard of five probe compounds, octane, heptanal, methyl hexanoate, nonanol and 2-decanone representing classes of compound previously observed in human saliva [157] and spanning a representative range of chemical functionality. 1.8 cm^3 of this standard solution was aliquoted into a 2 cm^3 headspace vial, this minimised the headspace in the vial and allowed liquid displacement during sampling with the SPE cartridge. The SPE cartridge was mounted on the spindle from the holder (Figure 3.5) and placed into the vial which was sealed and left to stand in a thermostatically controlled oven at 37 °C for 5 min at the end of which the SPE cartridge was removed, wiped with a lint free wipe to remove excess water, and placed immediately into a thermal desorption tube for analysis. Five replicate samples were acquired following this procedure and the results are summarised in Table 3.2 and Figure 3.7.

Table 3.2 Assessment of the reproducibility of the saliva sampler, *in-vitro.*

	Compound	*M.Wt.*	P_v	T_B	Q	t_r	*A*	RSD
1	Octane	114.14	1726	399	43	7.3	20	26
2	Heptanal	114.10	378	426	70	10.1	108	23
3	Methyl hexanoate	130.10	50	423	74	10.8	272	1.3
4	5-Nonanol	144.15	14	468	69	16.1	397	2.7
5	2-Decanone	156.15	33	484	58	19.4	260	5.5

Note. $M.Wt$, relative molecular mass; P_v, Vapour pressure /Pa at 25 °C (taken from Chemspider, www.chemspider.com); T_B, Boiling point / K; Q, Quantitation ion (m/z); t_r ,retention time / min; A, chromatographic peak area; and, RSD, relative standard deviation (%).

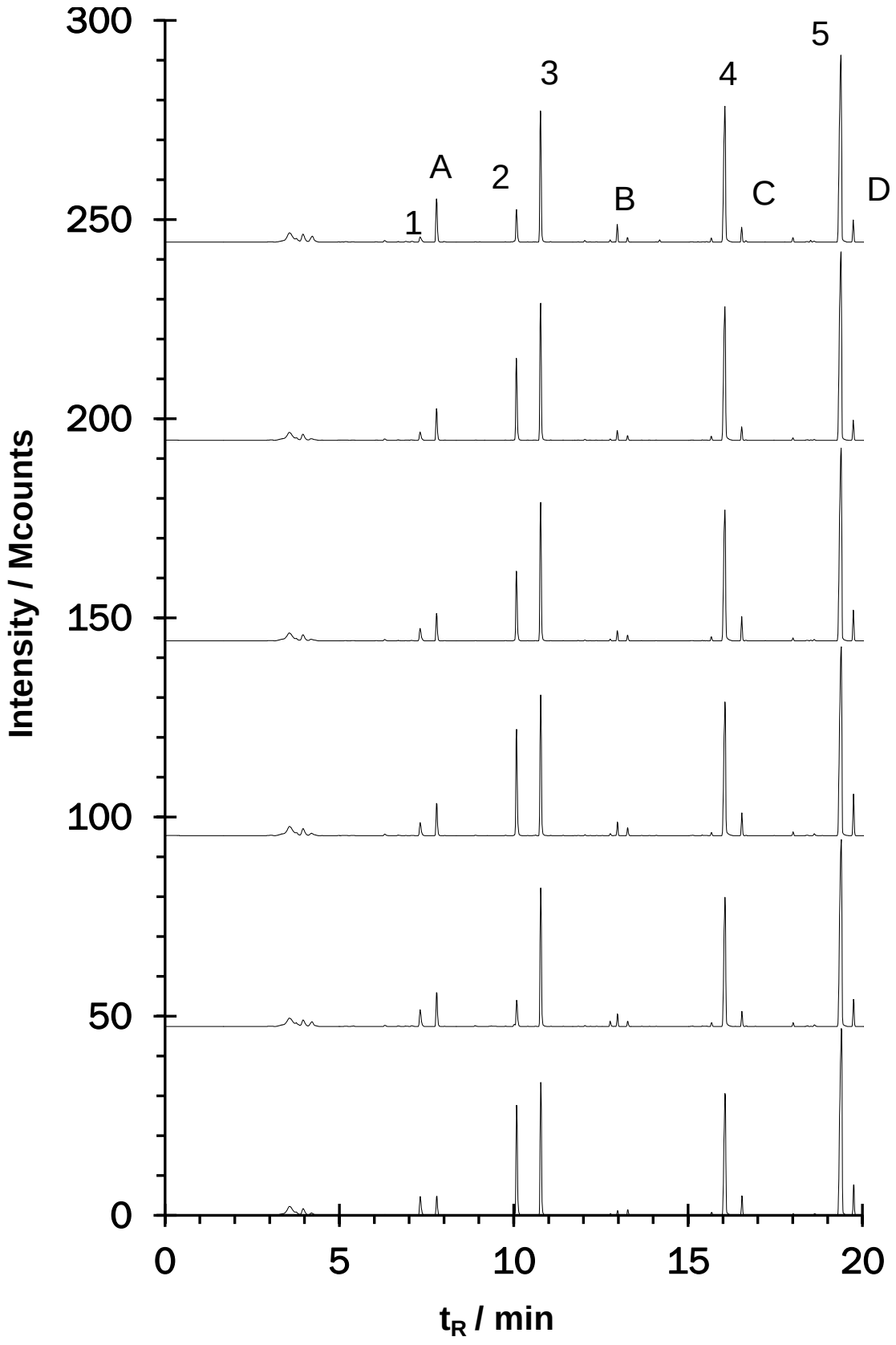


Figure 3.7 TIC chromatograms from in-vitro sampling with the saliva sampler. Six replicate 1.8 ml samples of an aqueous 1ppm standard of octane (1), heptanal (2), methyl – hexanoate (3), 5-nonanol (4) and 2-decanone (5) are shown. Also labeled are artefacts from the PDMS sampling medium (A-D).

The underlying processes controlling the recovery of the probe molecules into the PDMS phase include absorption, followed by diffusion and permeation as described in Section 2.2, and these will be a function of concentration, volatility, hydrophobicity and functionality/polarity. The most important predictive molecular characteristic appears to be vapour pressure. As the vapour pressure increases so does the relative standard deviation (RSD). The amount of material recovered is also inversely correlated to the vapour pressure with the size of the chromatographic response increasing logarithmically with reducing vapour pressure, see Figure 3.8. Such trends indicate that the most volatile compounds are highly susceptible to small variations in sampling procedures, most rapidly lost from the experiment and recovered with the lowest efficiency, a similar observation has been noted previously [111].

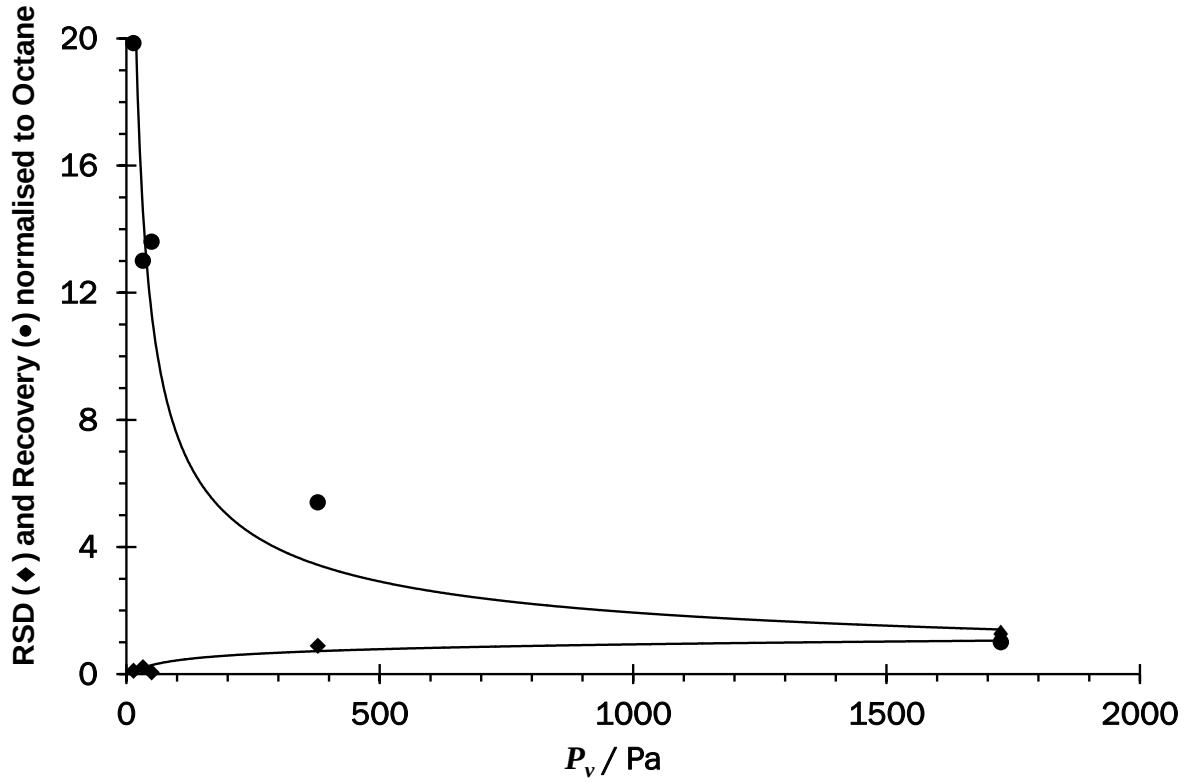


Figure 3.8 A plot of relative standard deviation (RSD) and recovery against vapour pressure for data from *in-vitro* reproducibility studies. Examination of the recovery data (circles) and reproducibility data (diamonds) in Table 3.2 reveals how vapour pressure predicts the recovery and reproducibility of VOC. These two plots show how RSD increases, and recovery reduces, with analyte vapour pressure. These data are plotted relative to those of octane to enable both trends to be viewed easily on the same graph.

Data for octane (Table 3.2), Pv: 1726 Pa at 25°C, A: 20, and RSD: 26%.

In-vivo reproducibility was assessed by taking six samples from the sub-lingual region of the mouth of a female participant using the procedure described above with a sampling-time of 5 min. The participant was allowed to rest for 1 min between each sample. The complexity of

VOC profiles obtained from human saliva is illustrated in Figure 3.6 which shows intensities ranging across two to three orders of magnitude and approximately 100 resolved and many more unresolved chromatographic peaks.

In common with data where the variance is significant, means have low values and negative responses are not possible the VOC profiles from saliva sampled *in-vivo* appear to be log-normally distributed. Indeed such log-normal distributions are often observed in biological systems; cell division of bacteria, latent periods of infectious diseases, permeability and solute mobility in plant cuticles for example [127] as well as antibody concentration in human blood sera[127] [158]. Eight randomised peaks selected from the study were assessed for normal and log normal distributions using the Anderson-Darling test and the cumulative distribution function appeared to fit a log-normal distribution better than a normal one. The "S"-shaped curves, linear probability plots, and symmetric distribution expected in normally distributed data were not observed. Figure 3.9 provides an example of this behaviour.

The Anderson-Darling test is a goodness of fit test based on the cumulative frequency function. It provides a measure of how well the data follow a particular distribution, the smaller the AD value the better the fit. The probability of obtaining these results when the distribution of the data is true is quantified as a *p* value. When comparing distributions to determine the best fit it is useful to compare both AD and *p* values; the distribution with the smaller AD and larger p values best fits the data. Table 3.3 gives example AD and p values for compounds from *in-vivo* saliva samples.

Table 3.3 Example AD and p values providing evidence for log-normal distributions in human saliva

	Normal Distribution		Lognormal Distribution	
t_R / min	AD	*p*	AD	*p*
5.2	0.336	0.364	0.169	0.882
18.3	0.930	0.007	0.417	0.214
20.3	0.336	0.364	0.213	0.733
32.7	0.311	0.428	0.158	0.904
38.9	1.081	<0.005	0.300	0.459
40.0	0.740	0.025	0.256	0.570
43.6	0.422	0.207	0.169	0.884
45.4	0.740	0.025	0.256	0.570

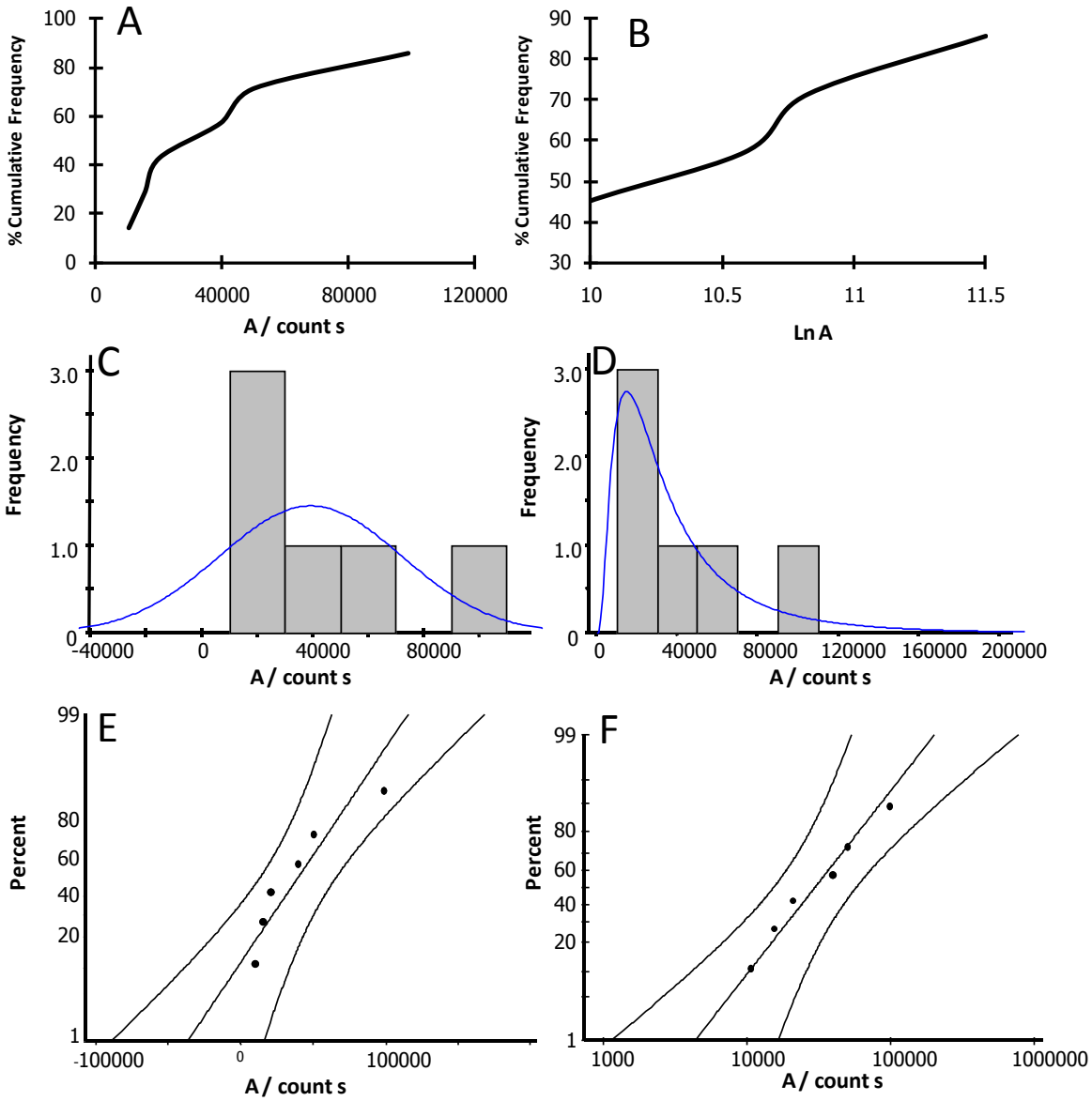


Figure 3.9 Example of evidence for log-normal distributions of VOC concentrations in human saliva. Cumulative frequency curves, for a benzoic acid ester, t_R = 46.3 min in Table 3.3, showing normal (A) and log-normal (B) distributions, histograms for normal (C) and log-normal (D) distributions and probability plots for normal (E) and log-normal (F) distributions suggest that VOC levels in the saliva of the participant studied better fit a log-normal than a normal distribution.

For the *in-vivo* reproducibility data six compounds were selected at random across the chromatogram and, where possible, identified from NIST mass-spectral matches as; propanoic acid, an unassigned entity, 2-phenoxy ethanol, n-hexyl salicylate, benzoic acid ester-1, and finally another benzoic acid ester-2. For these probe compounds the hypothesis of normality was rejected and a log-normal distribution was adopted on the basis that no significant departure from a log-normal distribution was observed for any them, such observations lead to the inference that the levels of VOCs in human saliva may better follow a log-normal than a normal distribution. A summary of the *in-vivo* reproducibility is given in Table 3.4.

Table 3.4 Assessment of the reproducibility of the saliva sampler, *in-vivo.*

	Assignment	Q	t_R	ln *A*	RSD
1	Propanoic Acid	74	5.2	7.62	18.2
2	Unassigned	106	18.3	5.77	15.7
3	2-phenoxy Ethanol	94	20.3	4.94	6.4
4	n-Hexyl Salicylate	120	32.7	6.68	9.9
5	Benzoic acid ester -1	123	43.6	10.29	8.8
6	Benzoic acid ester-2	123	45.4	10.27	9.4

Note. Q, Quantitation ion (*m/z*); t_R ,retention time / min; A, average chromatographic peak area; and, RSD, relative standard deviation (%).

The data from *in-vitro* and *in-vivo* studies are consistent with a methodology that performs in a predictable and reliable way. Importantly these observations highlight the priority of sample management in the analytical work flow, especially for the most volatile components within saliva samples, with recovery and reproducibility strongly influenced by the volatility (expressed as vapour pressure) of the prospective analyte.

3.3.2.2. *In-vivo* sampling time

Ten chromatographic peaks selected at random retention times across the profile were used to assess the effect of sampling time on analyte recovery *in-vivo.* The compounds were assigned identities based on NIST mass spectral database searches, and these tentative assignments have not been confirmed with standards. This discussion will focus on the observed sampling time dependencies of four example compounds, one from each of the retention time quartiles. Figure 3.10 shows a histogram presenting the chromatographic peaks for ethanoic acid, 6-methyl-5-hepten-2-one, heptadecane and, an unidentified alkylbenzoate.

Ethanoic acid was accumulated rapidly over the first 5 min of sampling. After this the rate slowed reaching a maximum between 5 and 10 min. A further increase in sampling time resulted in a reduced response. 6-methyl-5-hepten-2-one showed a similar trend of an accumulation to a maximum value followed by a reduction in recovery; in this case the maximum recovery was achieved with a sampling time of *ca.* 15 min. The less volatile heptadecane and the unidentified alkylbenzoate, present at significantly lower levels, were accumulated with a gradual increase in abundance over 10 min before tending to equilibrate with the sampler.

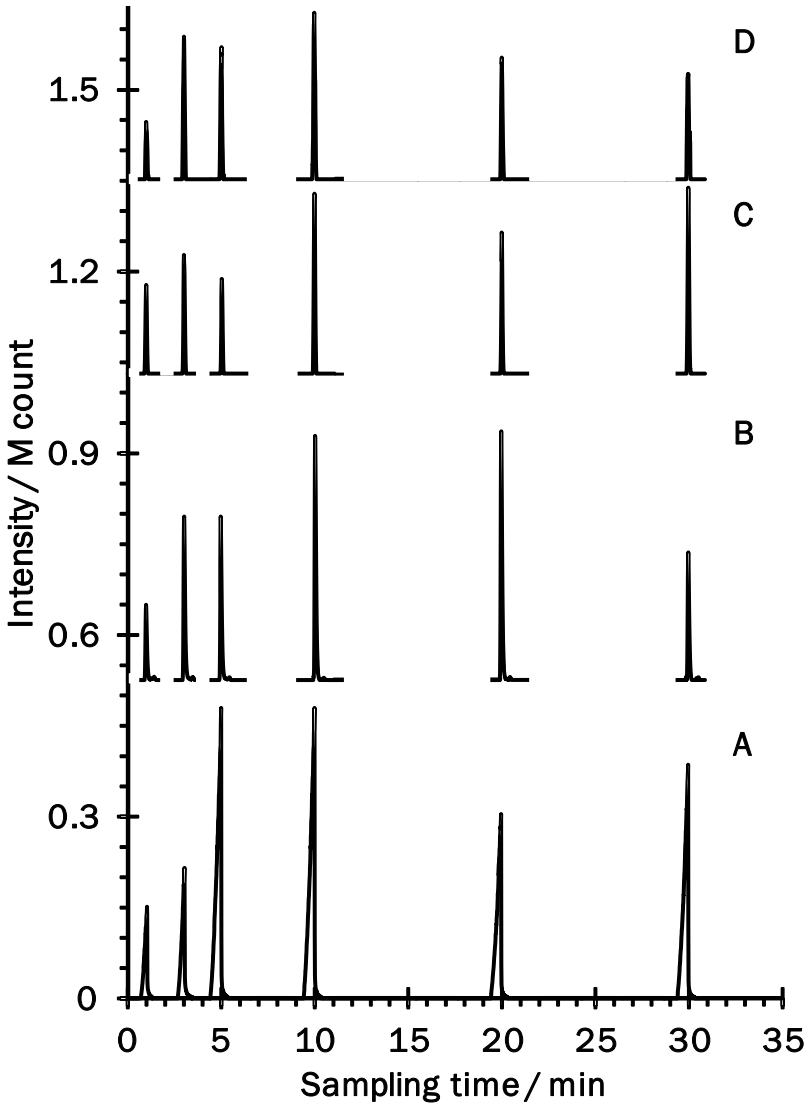


Figure 3.10 A histogram showing the effect of sampling time on the recovery of ethanoic acid (A, t_R 5.9min), 6-methyl-5-hepyen-2-one (B, t_R 25.1min), heptadecane at 10 x magnification (C, t_R 73.8min), and an unidentified alkylbenzoate at 100 x magnification (D, t_R 112.6min). The deconvolved peaks for these VOCs are shown against different sampling time.

The underlying physical chemistry and metabolic/catabolic processes governing these behaviours are likely to involve a combination of partitioning of VOCs from the saliva into the PDMS phase of the sampler cartridge, the continual release of metabolites/catabolites into the saliva and the kinetics of permeation and diffusion of the compounds into and out of the PDMS phase. Initially, VOCs in the saliva may be viewed as partitioning into the PDMS driven by concentration gradients, and more volatile species are likely to penetrate further and accumulate more rapidly in the sampling medium (PDMS). The kinetics of less volatile and perhaps lower concentration, compounds will be reflected in a slower accumulation. However, the partition coefficients for compounds of this nature are higher and so as sampling progresses the PDMS sampling phase moves closer to equilibrium with the surrounding saliva

and competitive adsorption / absorption processes would result in the displacement of the more volatile components, equation 3.1 is helpful in visualizing such a phenomenon [47].

Equation 3. 1

$$[i]_{PDMS} = \frac{K_i[i]_{saliva}}{(1 + K_i[i]_{saliva}) + \sum K_j[j]_{saliva}}$$

The equilibrium concentration of an analyte in the PDMS sampling phase ($[i]_{PDMS}$) may be expressed in terms of its partition coefficient between saliva and PDMS (K_i), the concentration of the analyte in saliva ($[i]_{saliva}$) and the sum of the products of the concentrations and equilibrium constants of the competing co-absorbed species ($\sum K_j[j]_{saliva}$).

The reduced recovery for the more volatile compounds with increased sampling time is likely to be due not only to competitive adsorption / absorption but to a combination of factors. Principle among which will be depletion of the surrounding saliva; absorption is an equilibrium process and initially the concentration gradient from the saliva into the PDMS is large, the more volatile species will accumulate rapidly, if subsequent release of such compounds into the saliva proceeds at a slower rate there will come a point at which the concentration gradient reverses and higher levels of the more volatile compounds are now in the PDMS. Continued sampling after this point will result in losses of these compounds from the sampler as the system shifts to re-establish equilibrium [47].

Figure 3.10 shows that selection of one sampling time for such a range of compounds that may be expected in a human saliva sample is not a trivial task. Optimal conditions for sampling will depend on the analyte under study and the composition of the matrix at a given point in time. The challenge for profiling VOCs in saliva is to develop a method that provides an accurate representation of as many compounds within the target range of the profile as possible. Conversely this also highlights the inherent selectivity of such techniques and the opportunity to 'tune' sampling methodologies for targeted analysis.

It is difficult to draw a conclusion as to the optimum sampling time from this study, the nature of the sampling does not permit simultaneous sampling and as such the in-vivo concentration may have changed from one sample to another, even with randomisation and careful experimental design.

Figure 3.10 indicates that 10 min appears to be an acceptable operational compromise for a sampling time, whilst still maintaining participant comfort, and this was the sampling time used for the rest of this study.

3.3.2.3. Comparison with expressed saliva

To provide a comparison of saliva profiles obtained from the SPE cartridge placed directly into the oral cavity (*in-situ* sampling) and from expressed whole saliva, a 10 participant (5 male, 5 female) double cross-over design was adopted. Each participant provided samples during two separate study visits where they gave *in-situ* and passive drool samples [47]. The study design is summarised in Table 3.5. 1.8 cm^3 of whole saliva was transferred to a 2 cm^3 headspace vial immediately after sampling and extracted using SPE cartridges as described for *in-vitro* sampling in Section 3.3.2.1. Both sample types were extracted for 10 min.

Table 3.5 Summary of the double-crossover sampling campaign adopted for the comparison of saliva samples collected *in-situ* with expressed saliva samples.

Sample order$_{(x-y)}$	F-02	M-03	M-04	M-05	M-06	F-01	F-07	F-08	M-10	F-09
1-1	I-S#01	I-S#03	P-D#01	I-S#09	I-S#13	P-D#15	P-D#17	P-D#19	P-D#23	I-S#25
1-2	P-D#02	P-D#04	I-S#08	P-D#10	P-D#14	I-S#16	I-S#18	I-S#20	I-S#24	P-D#26
2-1	P-D#05	P-D#11	I-S#33	P-D#21	P-D#39	1-S#37	I-S#31	I-S#27	I-S#35	P-D#29
2-2	I-S#06	I-S#12	P-D#34	I-S#22	I-S#40	P-D#38	P-D#32	P-D#28	P-D#36	I-S#30

Note: Sample order: x-y where x: visit number and y: sample number. The participant code designates gender (M/F) and their study identifier number. Each sample is designated as I-S: in-situ or P-D passive drool followed by the position in campaign sequence #. The order of the participants reading left to right designates the order of their recruitment to this study.

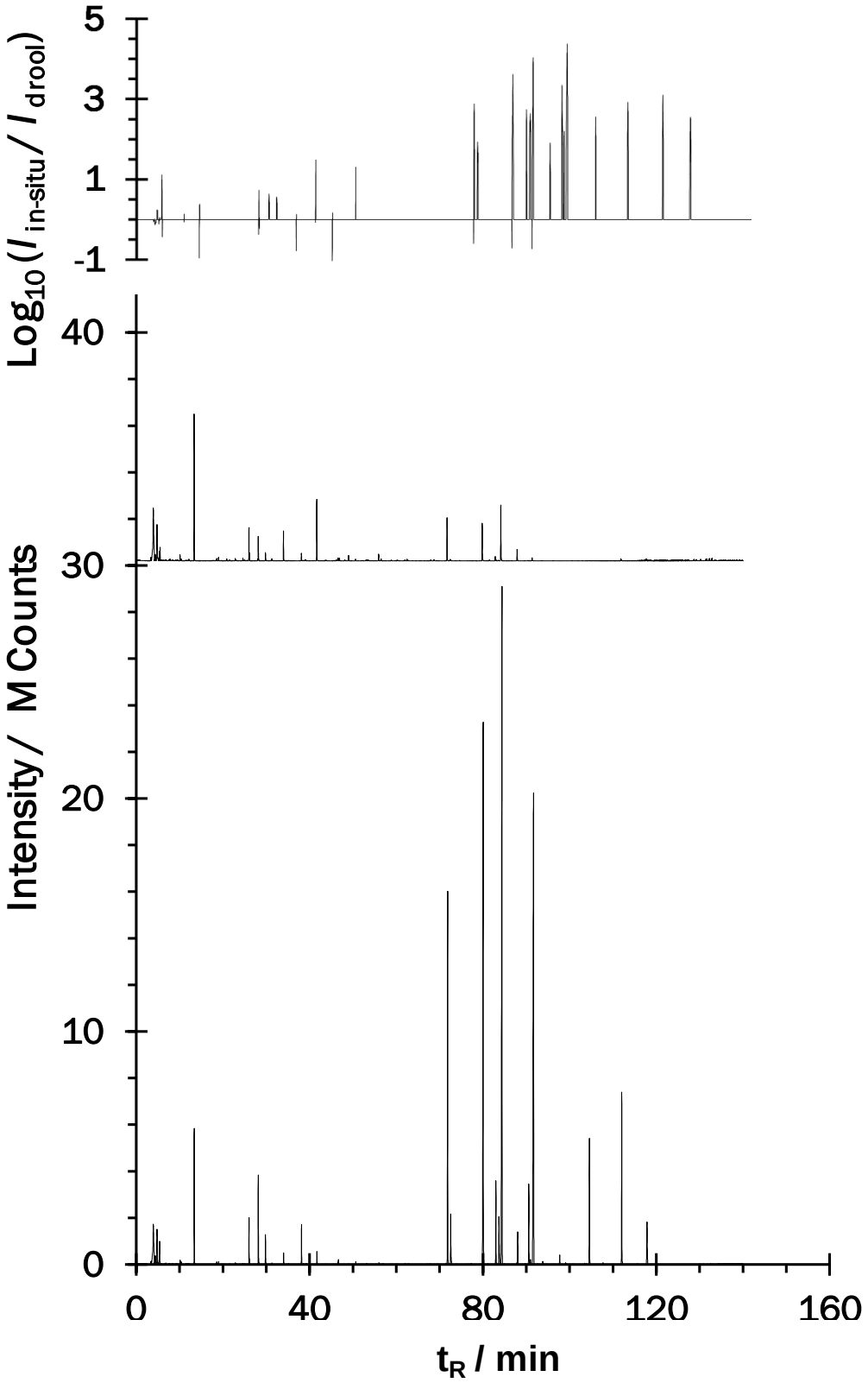


Figure 3.11 A comparison of the VOC profiles obtained from saliva samples from one participant. The bottom trace shows the profile achieved by in-situ sampling with a bespoke PDMS sampler. Offset at 30 M Counts is a profile from an expressed sample collected by 'passive drool'. The top trace shows the log of the relative responses for all the peaks with an intensity greater than 1% of the maximum.

Figure 3.11 shows that the chromatograms obtained from the two sampling techniques were fundamentally different, while there are many qualitative similarities, with 70% of components

present in both cases, the differences in intensity were marked. Somewhat surprisingly this was especially the case at higher retention times. Six compounds were selected to represent the volatility range across the profile and where possible tentatively identified from NIST mass spectral matches, Figure 3.12 shows box-whisker plots for these compounds with each sampling protocol.

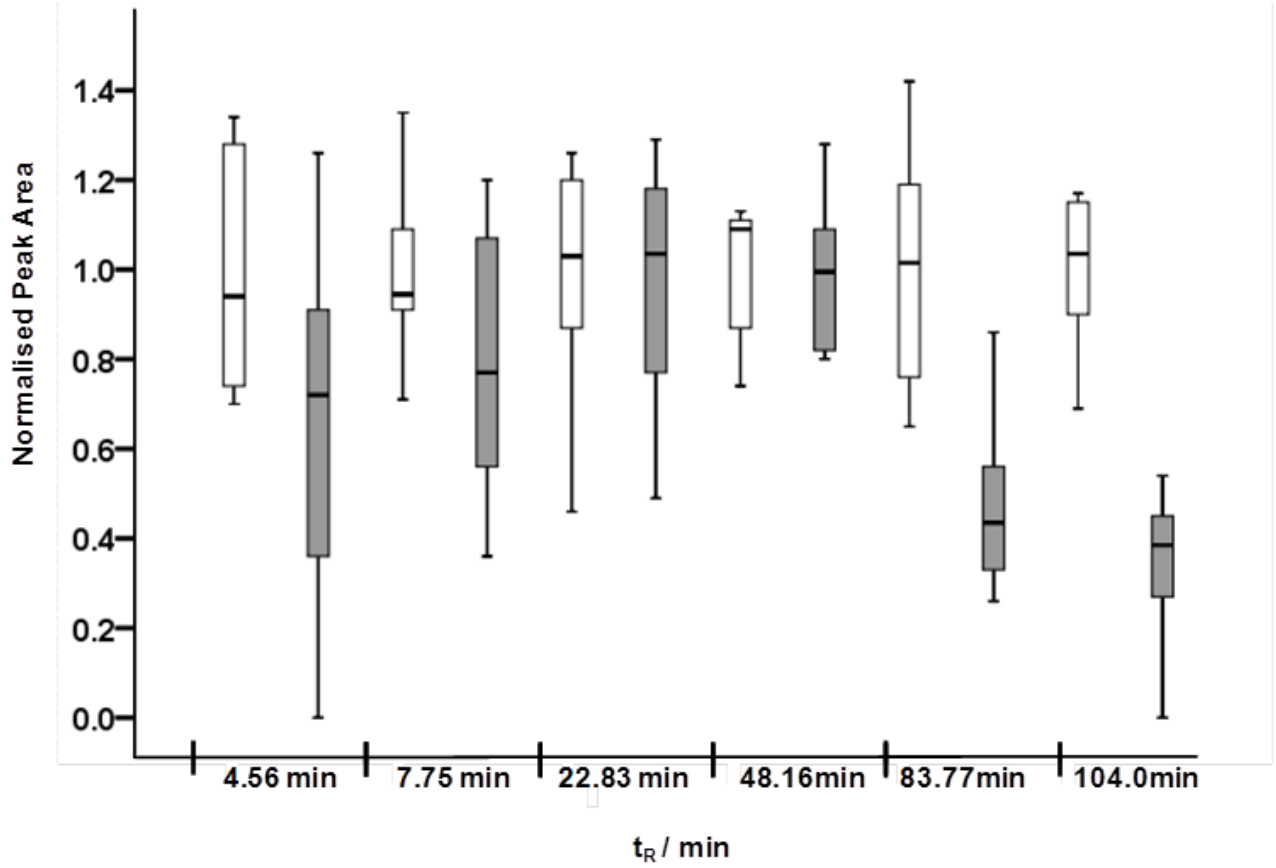


Figure 3.12 Box whisker plots showing the differences in saliva VOC profiles obtained by *in-situ* sampling and passive drool sampling in a double crossover study with 5 male and 5 female participants. 6 compounds were selected across the retention time range; acetone (t_R = 4.56 min), propanoic acid (t_R = 7.75 min), benzaldehyde (t_R = 22.83 min), indole (t_R = 48.16 min), n-hexadecanoic acid (t_R = 83.77 min) and an unidentified benzoic acid ester (t_R = 104.0 min). Peak areas have been normalized so that the mean of the *in-situ* samples is equal to 1 to allow all compounds to be presented on the same scale. At each retention time the white boxes show normalized peak areas for *in-situ* samples, the grey the same for passively drooled sampled. The higher recoveries and lower variability from the *in-situ* sampling is particularly evident at the extremes of the profile.

The two most volatile compounds, acetone (t_R = 4.56 min) and propanoic acid (t_R = 7.75 min), exhibit higher recoveries and lower variability when sampled *in-situ* compared to expressed saliva. The middle of the volatility range appears to be less affected with no notable difference in recovery or variability for benzaldehyde (t_R = 22.83 min) and indole (t_R = 48.16 min) between the two sampling techniques. The marked reduction in recovery for the higher molecular weight compounds is highlighted in Figure 3.12 with n-hexadecanoic acid (t_R = 83.77 min) and an unidentified benzoic acid ester (t_R = 104.0 min) exhibiting significantly lower recoveries when

sampled from expressed whole saliva. Higher recoveries of the least volatiles compounds from the *in-situ* sampler may be the result of contact between the PDMS and the surface membranes of the mouth or losses due to bacterial degradation in expressed saliva samples. Whilst every care was taken in this study to ensure rapid transfer of saliva from the collection vessel to the sampling vial for subsequent extraction (the time from the end of the saliva collection time to the start of SPE sampling was less than 5 min in all cases) it can take up to 10 min for some participants to produce 3 cm^3 of drooled saliva and headspace losses and bacterial degradation may be reasonably anticipated with passively drooled samples.

3.3.3. Summary of non-invasive, *in-situ* saliva sampling

This sampling technique was well tolerated by volunteers, simple, fast, reproducible and easy to administer and requires no specialist handling or storage techniques, making it more applicable to large scale field based sampling campaigns than collecting whole saliva.

An important element of this work was to develop a robust analytical workflow for saliva sampling that was compatible with other non-invasive, *in-vivo* VOC sampling and analysis methods, in particular breath and skin. In adopting this methodology care needs to be taken to ensure sampling times are carefully controlled and where possible matched to the volatility of analytes under study, this has been previously noted for *in-vivo* sampling of skin and for global profiling must represent a compromise between the most and least volatile analytes as well as constraints imposed by the study conditions and comfort of the participants. These observations highlight the inherent enhancements in sensitivity and selectivity that may be achieved by careful optimisation of these techniques for subsequent targeted studies and conversely serves as a reminder of the ease with which bias can be inadvertently introduced if this factor is not carefully managed.

Long term storage stability of these samples has not been addressed in this work principally as a result of a shortage of SPE cartridges preventing sufficient data points to provide statistically significant data. Short term storage of up to 24 hours at 4 ºC was verified with a series of 5 paired SPE cartridges sampled simultaneously, one of which was analysed immediately and the other stored for 24 hours. Qualitative assessments of the data showed the same profiles to be achieved for both samples with peak intensities normalised as a percentage of the most intense peak in the chromatogram, all tested peaks were within +/- 5% within sample pairs.

The tentative observation of log-normal distributions is potentially important and may have wider ramifications with other *in-vivo* measurement techniques. Characterising the inherent variability in saliva VOC profiles is not the focus of this thesis and the next logical step would be

to incorporate a follow up study with this as the main focus as part of a wider investigation into homeostasis in human saliva.

Comparison of *in-situ* sampling with expressed saliva has revealed that *in-situ* sampling provides more analytical information with lower intrinsic variability and indicates significant analytical utility for this form of *in-situ* sampling. Sufficient sensitivity and precision has been demonstrated to enable this sampling methodology to be included alongside existing breath and skin analysis techniques in VOC profiling and biomarker prospecting studies.

Subsequent studies in this thesis will apply the protocols established in this section for preparing, sampling and storing saliva VOC profiles from human participants to global profiling in pilot metabolomic studies to assess the utility of this form of saliva sampling alongside skin and other non-invasive sampling methodologies.

3.4. INSTRUMENTAL PARAMETERS

3.4.1. Thermal desorption

Thermal desorption of VOCs from PDMS samplers was achieved by two-stage thermal desorption, Markes International Unity 2, employing a Tenax TA / Carbograph 1TD cold trap for refocusing.

Direct desorption of PDMS samplers to obtain a representative profile of the absorbed VOCs from human skin or saliva must be an exhaustive process, that is to say it must achieve complete extraction of VOCs from the PDMS. Heating the PDMS in a flow of inert carrier gas sets up a dynamic equilibrium in which volatile analytes desorbed from the PDMS are instantly removed from its headspace. This effectively provides a constant headspace concentration of zero, maintaining the maximum concentration gradient from the PDMS into the carrier gas.

A desorption temperature of 180 °C was chosen as the optimum balance between efficient desorption and minimum bleed from the PDMS phase and the desorption time was evaluated between 2 and 20 min for both desorption efficiency and breakthrough of the most volatile compounds from the cold trap. To ensure that exhaustive extraction had been achieved PDMS samplers were 'redesorbed' to asses carryover Figure 3.13 shows an example skin VOC profile and the same Skin Patch analysed under the same conditions in the subsequent analytical run. Desorption efficiency at each desorption time was quantified by the % carryover of a set of randomly selected peaks across the profile and extraction was deemed exhaustive when less than 1% carryover was observed. For the Skin Patch 10 min desorption was required for quantitative extraction, whereas for the SPE cartridge 5 min was sufficient.

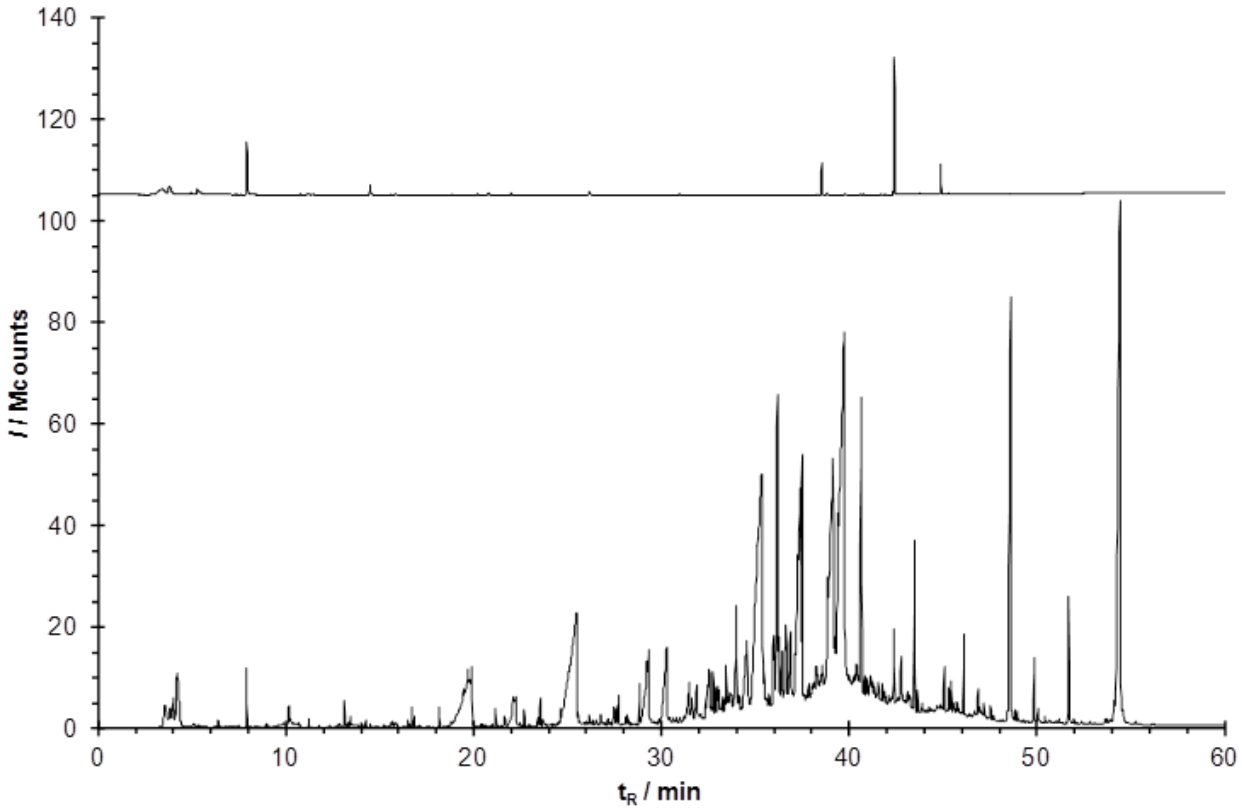


Figure 3.13 An example total ion current chromatogram (TIC) from TD-GC-MS analysis of a PDMS skin patch sample (bottom). Offset at 105 M Counts is a subsequent re-desorption of the same skin patch. Less than 1% carryover is exhibited for any compound not originating from the PDMS membrane itself. The formation of these PDMS derived compounds will be discussed in more detail in Section 3.5.3.

To ensure quantitative retention of the most volatile compounds on the cold trap the responses of acetone and acetic acid were monitored at each desorption time, if a reduction in response was observed with an increase in desorption time the breakthrough volume of that compound had been exceeded and the desorption time would need to be reduced. Due to the hydrophobic nature of both PDMS and the sorbents in the cold trap the cold trap could be held at sub – ambient temperatures without large volumes of water being retained and as a result no breakthrough was observed for either acetone or acetic acid with desorption times up to 15 min.

A summary of optimised thermal desorption method parameters employed in these studies is included in Table 3.6.

Table 3.6 A summary of the thermal desorption parameters used in the analysis of skin and saliva VOC samples.

Parameter	Skin Patch	SPE Cartridge	Unit
Prepurge			
Flow	30	30	$cm^3 min^{-1}$
Time	1	1	min
Primary Desorption			
Flow	50	50	$cm^3 min^{-1}$
Temperature	180	180	ºC
Time	10	5	min
Split Flow	0 (Splitless)	0 (Splitless)	$cm^3 min^{-1}$
Cold Trap			
Low temperature	-10	-10	ºC
Secondary Desorption			
Flow	2	2	$cm^3 min^{-1}$
Temperature	300	300	ºC
Time	5	5	min
Split Flow	0 (Splitless)	0 (Splitless)	$cm^3 min^{-1}$
Heating Rate	~100 (max)	~100 (max)	ºC s^{-1}
Flow path temperature	180	180	ºC

3.4.2. Gas chromatography – mass spectrometry

A HP 5890 gas chromatograph fitted with a 60 m long, 0.25 mm internal diameter, 0.25 μm film thickness DB-5 MS column operating in constant pressure mode was employed in these studies. To achieve a column flow of 2 $cm^3 min^{-1}$ during the initial stages of the temperature programme, thus ensuring a 2 $cm^3 min^{-1}$ cold trap desorption flow, the system was operated with a column head pressure of 29 psi.

Separation of VOCs was achieved using a temperature programmed GC method and each method was study specific, these are outlined in Table 3.7.

Mass spectrometric detection was achieved using a Fisons Trio 1000 quadrupole mass spectrometer, operating in scanning mode, with an electron ionisation source, operating in positive ionisation mode. Conditions are summarised in Table 3.7.

Table 3.7 A summary of the gas chromatography and mass spectrometry parameters used in the analysis of skin and saliva VOC samples.

Parameter	Level	Unit
GC Programme 1		
Initial Column Temperature	30	ºC
Initial Hold Time	0	min
Initial Heating Rate	2	ºC min^{-1}
Final Column Temperature	300	ºC
Final Hold Time	8	min
GC Programme 2		
Initial Column Temperature	40	ºC
Initial Hold Time	0	min
Initial Heating Rate	3.3	ºC min^{-1}
2^{ry} Column Temp	90	ºC
2^{ry} Hold Time	0	min
2^{ry} Heating Rate	2.5	ºC min^{-1}
3^{ry} Column Temp	140	ºC
3^{ry} Hold Time	0	min
3^{ry} Heating Rate	10	ºC min^{-1}
Final Column Temp	300	ºC
Final Hold Time	8	min
GC Programme 3		
Initial Column Temperature	40	ºC
Initial Hold Time	0	min
Initial Heating Rate	5	ºC min^{-1}
Final Column Temperature	300	ºC
Final Hold Time	8	min
Mass Spectrometer Conditions		
Scan Range	40 - 445	*m/z*
Scan Time	0.45	s
Interscan Delay	0.05	s
Electron Energy	70	eV
Source Temperature	200	ºC
Interface Temperature	300	ºC

3.5. DATA PROCESSING

VOC profiles from human skin and saliva are complex containing hundreds of resolved and unresolved compounds, with responses ranging over several orders of magnitude. This presents a significant challenge for establishing simple and robust automated data processing work flows. This section discusses the key factors associated with handling large complex data sets and describes the strategy adopted in these studies alongside the optimisation of critical parameters for each step in the workflow.

3.5.1. Dynamic background compensation (DBC)

In these studies DBC and mass spectral deconvolution were achieved using TargetView (ALMSCO International, Llantrisant, UK) as described in Section 2.6.2. Important parameters to consider when using DBC algorithms are the peak width and noise counts settings, the noise counts value is an absolute value below which spectral peaks are attributed to noise. More importantly for skin and saliva samples it may be that different peak width values give optimal peak shapes for different classes of compound. Figure 3.14 shows an extracted ion chromatogram for octanoic acid and octanal on a DB-5 column; used in this study. The shape of the octanoic acid peak is due to its higher polarity relative to the column stationary phase and is an unfortunate compromise when using one column for a wide range of analytes.

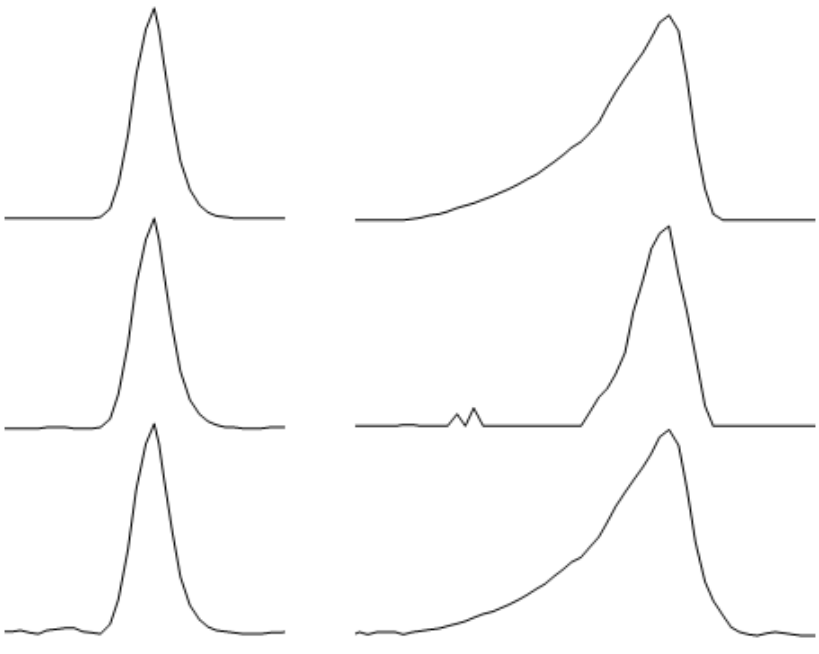


Figure 3.14 A demonstration of the effect of automated data processing parameters on typical skin VOC compounds. The different peak shapes in the extracted ion chromoatograms for octanal (m/z 56, left) and octanoic acid (m/z 60, right) are shown in the raw data (bottom trace). The middle trace shows dynamic background compensated data with a peak width setting of 10 seconds and the top a peak width of 30 seconds. The truncation of the octanoic acid peak with the 10 second peak width setting is evident.

Figure 3.14 shows octanoic acid and octanal before any processing and after DBC with a peak width of 10 seconds and 30 seconds, the truncation of the acid peak is evident with the smaller window and is an important reminder that optimisation of automated data handling techniques is equally as important as optimisation of instrumental parameters.

3.5.2. Mass spectral deconvolution

As with DBC the input peak width parameter in TargetView can have a significant effect on the resultant deconvolved spectrum and careful optimisation of this parameter is essential to achieve accurate spectra. Using a series of test compounds identified in a series of skin samples and previously reported in literature to be present in VOC profiles recovered from human skin [41] [83] [96] the effect of peak width on the accuracy of deconvolved spectra was tested using the NIST forward spectral match as a measurement of accuracy. Samples were chosen so that a representative concentration range of each compound was assessed and Table 3.8 summarises the variability in peak widths observed across the 10 data files.

Table 3.8 A summary of the variation in baseline chromatographic peak widths between classes of compound in 10 skin VOC samples.

	Peak width / s							
	AA	B	D-L	1-D	I	H	n-HA	S
1	33	15	4	9	6	5	48	33
2	35	10	6	7	7	5	60	35
3	26	8	9	13	6	6	90	24
4	25	12	5	12	8	5	51	27
5	20	13	10	8	5	3	102	31
6	41	18	12	9	7	4	98	35
7	38	20	13	10	6	5	100	38
8	27	15	5	11	4	7	75	20
9	34	6	6	13	8	6	63	22
10	30	10	7	10	7	5	72	21
Mean	30.9	12.7	7.7	10.2	6.6	4.8	75.9	28.6
Stdev	6.5	4.4	3.1	2.0	1.3	1.1	20.5	6.6
%RSD	20.9	34.6	40.6	20.0	19.2	22.9	27.0	23.1

Key: AA, Acetic Acid; B, Benzaldehyde; D-L, D-Limonene; 1-D, 1-Decanol; I, Indole; H, Hexadecane; n-HA, n-Hexadecanoic Acid; S, Squalene.

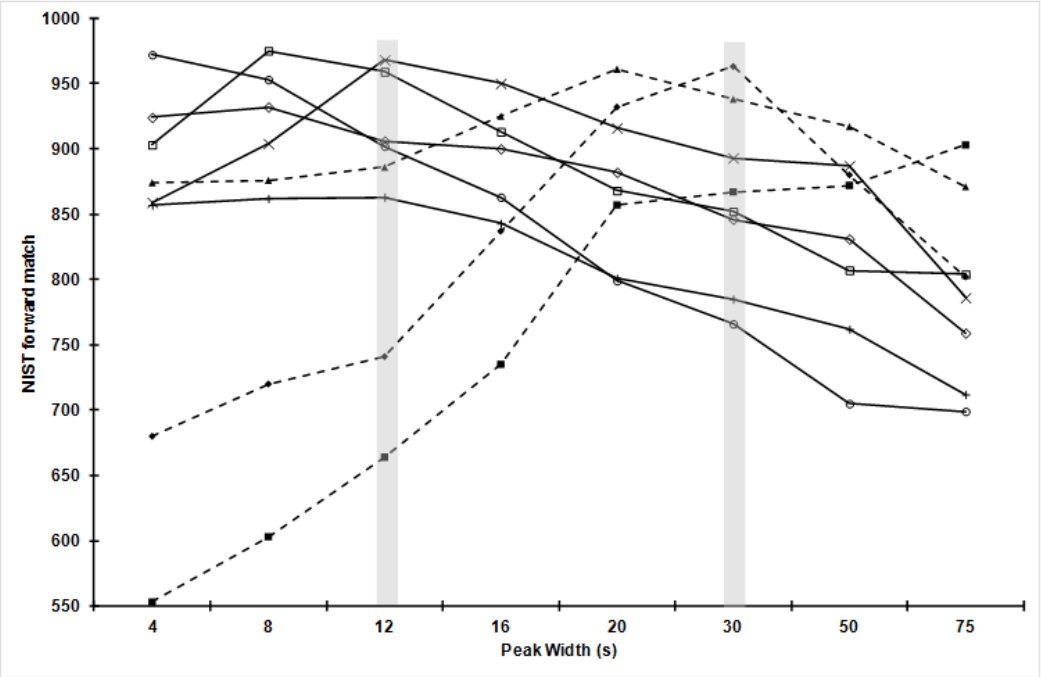


Figure 3.15 The effect of peak width on the accuracy of mass spectral deconvolution in skin VOC samples. The average forward mass spectral match generated by NIST for each of the 8 compounds and 10 samples summarised in Table 3.8 plotted against the peak width used for deconvolution shows two general trends. The dashed lines represent compounds with average peak widths >25 s and the solid lines those <25 s, the optimum peak width for each group is highlighted with grey shading.

The difference in peak widths and the large concentration range of compounds reported to be recovered from human skin pose serious difficulties for automated deconvolution. The results from interrogating the data with different peak width values indicate that, for the majority of compounds, increasing the peak width window has no marked effect on accuracy of deconvolution until a peak width of 16 s, these are represented with solid lines in Figure 3.15, and this observation appears to hold for all compounds exhibiting Gaussian peak shapes. The broader peaks however appear to be greatly affected by utilising too small a peak width, with key ions being removed from the deconvolved spectrum or classified as 'uncertain'.

In metabolomic studies it is simply not feasible for an analyst to optimise deconvolution settings for every peak in every chromatogram. To allow a degree of automation a compromise was employed in these studies by which peaks exhibiting Gaussian distribution were deconvolved with a peak width of 12 s, those that were not Gaussian in nature were found to be predominantly volatile fatty acids and primary amines and for these a peak width of 30 s was employed to obtain a deconvolved spectral list. Whilst this method still requires a significant degree of manual intervention it allows creation of accurately deconvolved spectral lists for inclusion into a database, these lists will ultimately provide quantitation and qualification ions for integrating raw data.

3.5.3. Chromatographic alignment

In these studies a homologous n-alkane standard series was used to generate a primary retention index ladder. By definition each n-alkane has a retention index equal to its carbon number multiplied by 100.

Table 3.9 A list of the standards used to create a primary retention index for chromatographic alignment of skin and saliva VOCs. This table lists each standard with an example retention time and the assigned retention index value.

Standard	t_R / min	RI
Octane	7.43	800
Nonane	10.15	900
Decane	13.3	1000
Undecane	16.55	1100
Dodecane	19.7	1200
Tetradecane	25.57	1400
Heptadecane	33.22	1700
Eicosane	39.64	2000

This standard solution was analysed as a quality control standard every 6 samples and assessed for any reductions in response, changes to the shape of the overall profile – i.e. loss of the lighter or heavier compounds, and any significant shifts in retention time.

Retention indexing is usually achieved by the addition of internal standards to a liquid sample. Rather than add a series of internal standards to PDMS samplers, not a trivial task, and then validate such a method, the ubiquitous series of siloxane compounds stripped from the PDMS sampling medium during thermal desorption was employed as internal markers. A secondary retention index ladder was thus created using these siloxane compounds, example TICs for primary and secondary retention index ladders are shown in Figure 3.16.

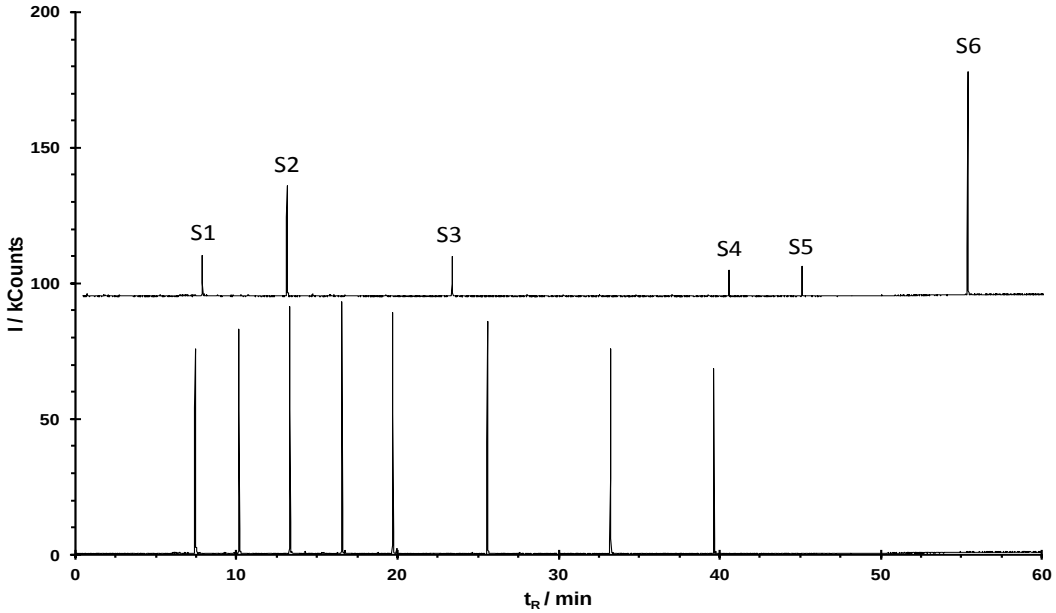


Figure 3.16 Bottom: Primary retention index ladder of n-alkane standards. Top: An example blank skin patch, S1-S6 are siloxane compounds used to create a secondary retention index ladder. The secondary retention index ladder, calculated based on the primary ladder, is used as an internal standard series for chromatographic alignment of skin / saliva VOCs.

Figure 3.17 shows the mechanism of thermal degradation of the PDMS phase to form one of the siloxanes forming the secondary retention index ladder, S1. The final compound eluting in both skin and saliva samples was found to be the skin lipid squalene, as this was present in all samples squalene was used as the final retention time marker for retention index alignment, Table 3.10 lists the internal retention index markers.

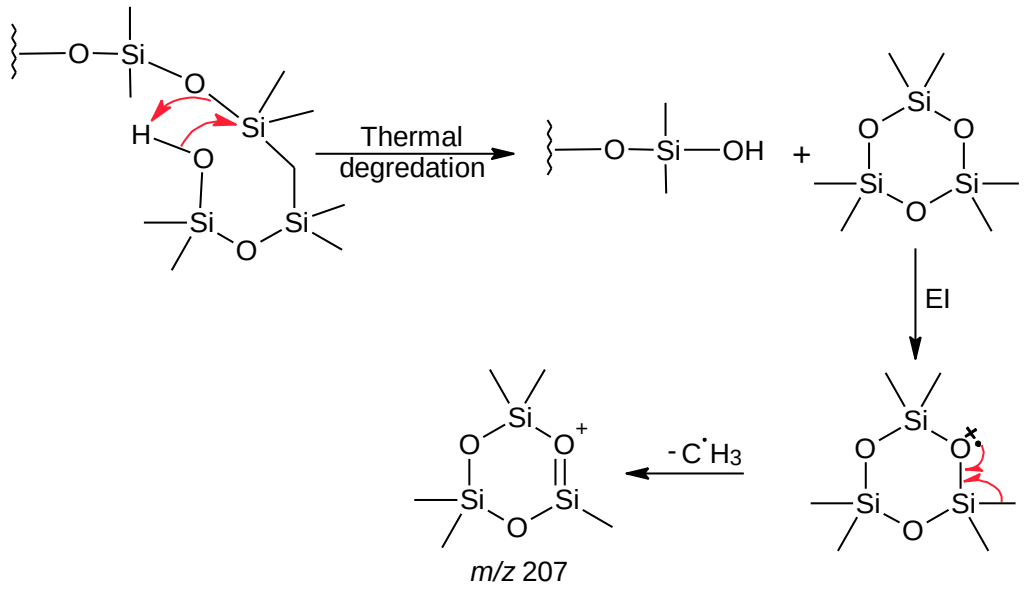
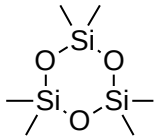
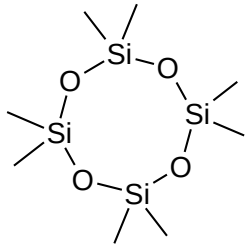
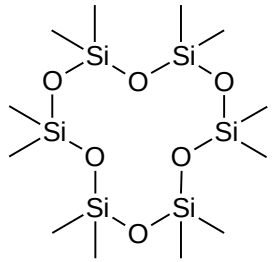
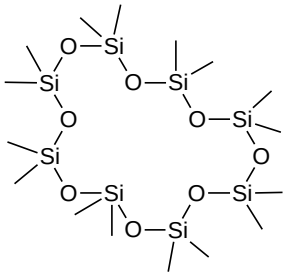
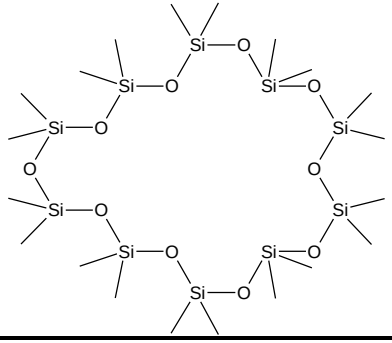
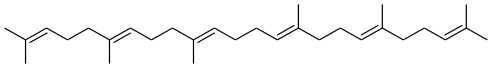


Figure 3.17 Mechanism showing the thermal degradation of a polydimethyl siloxane (PDMS) membrane and its subsequent electron ionisation and fragmentation to give *m/z* 207, a commonly observed siloxane from PDMS samplers and gas chromatography column stationary phases. Siloxanes such as this are used in these studies as internal retention time markers. This mechanism has been reproduced from reference 128.

A secondary retention index ladder was calculated from the primary index of n-alkane standards to assign retention indices to these ubiquitous siloxanes, this calibration was plotted for each sample. The retention index value for a specific VOC within a sample was then obtained by interpolating the respective retention time to the linear regression generated from a plot of retention time versus secondary retention index of the ubiquitous siloxanes as shown in Figure 3.18.

Table 3.10 The five ubiquitous siloxanes and skin lipid squalene used as internal retention indexing markers in skin and saliva VOC profiles. Each marker is defined by retention time (GC parameters 3), retention index, mass spectrum and structure.

Name *RI*, t_r / min, mass spectrum		
Hexamethylcyclotrisiloxane (S1) 796, 7.9, 207_208_149_209_191 	Octamethylcyclotetrasiloxane (S2) 987, 13.1, 281_282_283_207_133 	Dodecamethylcyclohexasiloxane (S3) 1361, 23.4, 73_429_341_430_147 
Hexadecamethylcyclooctasiloxane (S4) 1988, 40.6, 355_73_221_147_356 	Eicosamethylcyclodecasiloxane (S5) 2149, 45.1, 73_281_147_503_221 	Squalene (S6) 2491, 55.4, 69_81_41_95_68 

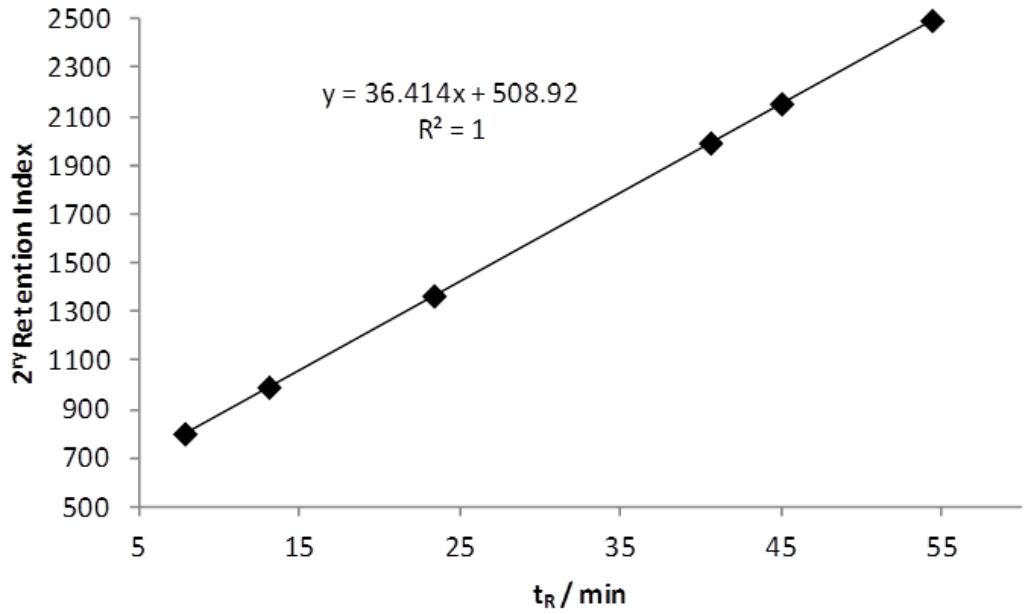


Figure 3.18 Example retention time to secondary retention index calibration based on the six compounds in Table 3.10. This calibration curve was plotted for each VOC sample to convert retention time to retention index thus aligning all samples chromatographically.

3.5.4. Generation of a VOC matrix and multivariate analysis

The penultimate step in the data processing workflow was incorporation of the compound information into a matrix for multivariate analysis (MVA). The variables for a matrix were given unique identifiers based on their calculated secondary retention index values (RI) and the 5 most intense peaks from deconvolved mass spectra taking the form RI_Base Peak_Fragment 1_Fragment 2..... and so on. Each separate sample became an observation thus creating the template for the matrix as detailed in Section 2.7. Exogenous compounds were removed from the matrix if they were found to be present in sampler blanks, typically 200 to 300 VOCs were found in a skin sample after deconvolution and exogenous removal.

The matrix was then populated with peak areas calculated as the area under the curve for the extracted ion chromatogram of the designated quantitation ion for each variable. In the majority of cases this was the base peak but where this did not offer sufficient selectivity a different ion was chosen for quantitation. Integration was performed on raw data using MassLynx (Waters, Manchester, UK). If the peak area for a compound was below the detectable level a value of zero was entered into the matrix.

Once populated the VOC matrix was imported to SIMCA-P v.12 (Umetrics, Umea, Sweden) for multivariate analysis. To give equivalent weighting to all the components in the matrix pareto-scaling was applied. This divides the peak area of each individual component in the matrix by the square root of the standard deviation of the peak areas for that variable set [159-160] and

compensates for the large range in response between variables and assign equal significance to every variable, independent of relative magnitude.

In Section 2.7 the combination of supervised and unsupervised statistical models was described. Initially, partial least squares discriminant analysis was applied to the matrix in which the observations had been divided into classes. An S-plot was generated from this which graphically represents all of the variables in the matrix distributed through the S-plot co-ordinates according to the probability that they contribute to the separation between the classes. The variables identified from the S-plot as those with the highest probability of contributing to the variance between classes were then copied to a new matrix which was analysed using unsupervised principle component analysis (PCA). If separation between the classes was observed in the unsupervised model from these variables they were listed as candidate markers and more detailed inspection of the data was then undertaken to test this finding.

Candidate markers from MVA were identified on two criteria: a satisfactory match between the denconvolved mass spectrum, generated by TargetView, and the respective entry in the NIST MS library (NIST 11) and, a satisfactory match between the estimated Kováts retention index value obtained from NIST and the calculated retention index value based on a series of hydrocarbon standards as described in Section 3.5.3.

A spectral match was deemed satisfactory if forward and reverse match factors gave values greater than or equal to 750. A retention index match was deemed satisfactory if the observed retention index value was within 50 retention index units (RIU) of the published estimate. Where possible these tentative identifications were confirmed with pure standards.

3.6. CONCLUSIONS

It is evident that applying non-invasive sampling protocols to complex matrices for global VOC profiling in metabolomic studies is not straightforward. Suppressing bias within the sampling, analysis and data handling techniques presents a significant challenge. The research described in this Chapter sought to characterise and optimise the key parameters for sampler design and sampling methodology, recovery of sampled VOCs by thermal desorption, separation and detection and data preparation and processing. The result is a work flow for global profiling of VOCs from human skin and saliva.

Whilst this work flow requires a significant level of manual intervention from the analyst it presents the first real step towards a robust and valid protocol which encompasses all stages from sampling through to statistical modelling for these complex and challenging samples. Continual evaluation, enhancement and validation of this work flow will be a dynamic and on-

going task over the course of many studies and research cycles; the subsequent Chapters present its first application to pilot metabolomic studies and provide a critical evaluation of the utility of skin and saliva sampling alongside the current forerunner in non-invasive sampling, breath analysis.

In these early stage pilot studies healthy participants were recruited and subject to stress interventions to act as a surrogate for disease state, rather than using unproven methodologies with precious samples from un-well individuals. These studies may also provide useful insight into the potential confounding factors due to psychological and physical stress in patients.

4 THE EFFECT OF INDUCED PSYCHOLOGICAL STRESS ON VOC OF YOUNG ADULTS: A PILOT STUDY

4.1. INTRODUCTION

This chapter presents the first application of the Skin Patch methodology to non-targeted metabolomic profiling following the workflows established in Chapter 3. The intent was to critically evaluate the potential of the Skin Patch approach as a non-invasive sampler for *in-vivo* human VOC measurements. To do this the relative merits of skin and breath sampling were explored with parallel samples taken from participants subject to a controlled intervention designed to induce psychological stress. Candidate markers for stress identified from skin VOC profiles were compared against those from breath samples.

4.2. STRESS

'Stress', as it is understood in modern biology, was first discussed by Hans Seyle who described it as the failure of an organism to respond appropriately to perceived threats, whether emotional or physical [161]. Seyle proposed 3 stages of coping that the body undertakes to confront this threat; alarm, adaptation and finally exhaustion. Alarm is the initial response to the identified stress causing the hypothalamus to activate the pituitary gland, which in turn activates the adrenal glands to release adrenaline, nor-adrenaline and cortisol. The autonomic nervous system is also activated which leads to a number of physiological responses including increased heart rate and blood pressure, faster and shallower breathing and sweating. The liver responds by releasing sugars, cholesterol and fatty acids into the blood to supply energy to the muscles for the 'fight or flight' response. Muscles tense for action and the blood clotting ability increases to prepare for potential injury [162].

Adaptation follows in which a resistance to the stressor is built and the body remains mobilised to fight. Finally if the stress is prolonged, exhaustion occurs at which stage the bodies resources are depleted making it more vulnerable to stress related conditions [162].

These physiological responses indicate an alteration in metabolic state. Significant research effort has focused on calibrating these responses to be indicators of stress [163]. A study investigating the incidence of anxiety and depression in patients suffering from chronic obstructive pulmonary disease (COPD) suggests that comorbid psychological problems increase the functional disability of the patient, reducing quality of life. The authors propose psychological characteristics to be important variables that interact with physical symptoms and recommend that psychosocial variables are taken into consideration when treating patients suffering from COPD [164]. The observations in this study focused on the metabolic changes associated with stress and addressed the question:

are there stress sensitive compounds detectable in VOC profiles, and if so what implication does this have for putatative biomarker discovery?

The effect of stress on VOC profiles associated with breath and skin have yet to be established, and there may prove to be significant confounding factors in the disease marker discovery process. Conversely if biomarkers for stress exist in such samples the following question arises:

is it possible to objectively monitor stress, or distress, in those unable to communicate effectively to relatives and medical staff?

Significant research is currently focused on non-invasively identifying markers for chronic diseases, including COPD, in exhaled breath, skin, urine and saliva [34] [57] [121] [165]. The apparent higher prevalence of 'stress' in patients suffering from such diseases compared to healthy controls may contribute significant confounding factors and generate a false suite of candidate markers from such studies. This study therefore induced a stress response in young adults by employing a PASAT intervention and then evaluated changes in the skin VOC profile associated with the stress event.

Results from the concurrent breath study, published by Turner *et. al.*, reported 6 stress breath marker candidates. Levels of indole and 2-methylpentadecane were observed to increase with stress, whilst benzaldehyde, 2-hydroxy-1-phenylethanone, 2 ethylhexan-1-ol and an as yet unidentified terpene compound were shown to decrease with stress [62].

4.3. SAMPLING CAMPAIGN

20 healthy non-smoking adults (10 male and 10 female) were recruited from Loughborough University staff, students and social networks in the age range 19-26. The study followed a randomised cross-over design with participants attending two sampling sessions. In one they were asked to sit comfortably and listen to classical music, this was denoted as the 'neutral' intervention. In the other session, participants were asked to undertake a mental arithmetic test known as the Paced Auditory Serial Addition Task (PASAT), the 'stress' intervention.

The data presented in this chapter forms part of a wider study devised by the author in collaboration with Pareen Patel, Matthew Turner and Aditya Malkar. The PASAT test was provided by Dr Stephan Bandelow and Dr Louisa Edwards and administered by Toni McKay and Elliot Poole, all of the School of Sport, Health and Exercise Sciences at Loughborough University (SSHES).

4.3.1. Physiological measurements

Heart-rate (HR) and blood-pressure (BP) measurements were recorded using a Datex Ohmeda S/5 patient monitoring system. Heart rate was monitored via a finger clip attached to the index finger of the participants' left hand, a Critikon 22-33 cm Dura-Cuf blood pressure cuff was applied to the upper left arm. At each time point heart rate, systolic blood-pressure, diastolic blood-pressure and mean arterial pressure were recorded. Upon arrival 3 replicate measurements of the participants' blood pressure and heart rate were taken at 2 minute intervals to provide a baseline level, a further 12 readings were taken over the course of each experimental session at 12, 16, 19, 23, 30, 42, 45, 48, 49, 57, 60, 65 and 71 min.

4.3.2. Paced Auditory Serial Addition Task (PASAT)

The PASAT was first developed by Gronwall in 1977 to monitor the recovery of patients who had sustained mild head injuries [166]. The test involves single digits presented to the participant via an audio feed. The participant is required to verbally add each newly presented digit to the one immediately preceding it. Since its development to monitor recovery, the PASAT has found application as a psychological stressor in several studies. [166] [167].

In this study the PASAT was used to induce psychological stress. The protocol follows that described above with incorrect answers, and pre-defined correct answers, highlighted with the use of a loud horn to disorient and further stress the participant.

The participants were subjected to the neutral condition on their other study visit, this involved sitting in a quiet room listening to a collection of classical music provided by SSHES comprising four instrumental pieces; Truman Sleeps by Burkhard Dallwitz, Guitar Flute & String by Moby, Gabriel's Oboe by Ennio Morricone and Suite Bergamasque: Clair De Lune. The participant's received no input from the researchers during this time. Both interventions were 11 min in duration and the participants' blood pressure and heart rate was recorded at 4 time points during each.

4.3.3. Skin sampling

4.3.3.1. Selection of sampling site

The skin is a large complex organ adapted to perform a range of functions across different locations of the body and as a result the levels and classes of compounds emanating from the skin are likely to vary with body site. Careful consideration of sampling area was required to ensure any effects on the skin VOC profile due to the stress response were captured.

Although the main function of sweating in humans is thermal regulation, sweating from the palms often occurs when an individual is subject to emotional stimuli such as fear or mental strain [168]. Apocrine sweat glands are stimulated by emotional stress and open into hair follicles in body areas such as the axillae, eyelids and forehead. Eccrine glands on the palms of the hands and soles of the feet also respond to emotional stimuli [169] [170].

Emotional sweating has been evaluated in several studies as a measure of mental strain; measurements are usually based on electrodermal activity, employing techniques such as skin conductivity, which measures the resistance of skin to the passage of an electric current, known as the galvanic skin response [171][172]. One such study measured the electrodermal activity across the palm during three activities; arithmetic calculation to induce mental strain, a hand-grip exercise to induce physical strain and a deep breathing exercise to induce relaxation. All subjects showed a marked increase in palmar sweating during oral explanation of all three tasks, which returned to baseline before beginning the exercise. Subjects also showed an increase at the beginning of each exercise, whatever the stimulus, highlighting the importance of allowing time for equilibration to such interventions and the nervous response simply associated with being presented with such a task in a research environment. The palmar sweating response returned to baseline very quickly after beginning the deep inspiration exercise, but showed a continued elevation above baseline with the physical stimulus and gave an unstable high response to the mental strain exercise, indicating its applicability to assessment of mental strain [171]. It is also important to observe from this study that sweating responses to the mental arithmetic tasks reduced with repetition, indicating learned behaviour and reduced effectiveness of the task over time.

Kamei *et al* described sweating as a good indicator for surprise and emotional stress in their study on physical and emotional stress induced sweat secretions in the human palm and forehead [169]. By measuring the rates of sweating on the palm and forehead during a set of interventions the authors were able to conclude that the secretion of sweat is dependent on the way the individual absorbs stress, and propose the palm or forehead as locations for monitoring the human body under stress [169].

Investigations into the emotional sweating responses of medical students during a practical examination were conducted to determine whether forehead sweating could be applied, as a substitute for palmar sweating, for the measurement of mental strain when both hands were required for the task [173]. The majority of students monitored showed bursts of sweating during particularly difficult sections of the task and students who failed the first attempt showed elevated sweating during the second. Careful attention was paid to the ambient temperature and humidity during the experiment leading to the conclusion that increases in

forehead sweating were directly related to the emotional stress experienced by the participant [173].

A review of the literature has established that emotional stress is accompanied by a sweating response in certain areas of the human body. Based on this it can be proposed that the VOC profile associated with the skin in these regions will change during emotional stress. Several studies have indicated the potential for the forehead as a location for monitoring emotional sweating due to mental strain [171-173]. As the palms and axillae were inaccessible due to other sampling constraints in this study the forehead was chosen for sampling of skin VOCs.

4.3.3.2. Skin sample collection and analysis

Skin VOC profiles were recovered using the PDMS Skin Patch as described in Section 3.2, the Skin Patch was located centrally on the participant's forehead with a sampling time of 30 min. Sampled Skin Patches were then sealed into empty, inert coated stainless steel 3.5" x ¼" thermal desorption tubes and stored at -80 ºC until analysis. The analytical instrument parameters employed in this study are detailed in Tables 3.6 and 3.7, GC programme 2 was used in the analysis of both skin and breath samples.

As participants were asked to fast on the morning of each study visit and each visit was of approximately 1.5 hr duration only two study visits were scheduled per day. The first visit commenced at 9 am and the second at 11 am so the sampling campaign would span a minimum of 20 working days and in fact was a total of 6 weeks in duration. As discussed in Section 3.2.3 a skin sample can be confidently stored at -80 ºC for a maximum of 21 days with the current protocols and so the sampling and analysis campaigns were run concurrently.

4.4. RESULTS AND DISCUSSION

4.4.1. Blood pressure and heart rate

Average observations from collated heart rate and systolic and diastolic blood pressures clearly show responses to the PASAT and neutral interventions, shown in Figure 4.1. An autonomic nervous system response to the PASAT intervention is evident when observing both BP and HR whereas no discernible change was observed for the neutral intervention, providing compelling evidence for the applied stressor eliciting the predicted physiological responses.

4.4.2. Physiological response score

The participants' physiological responses to the PASAT (PSR_P) and neutral interventions (PSR_N) were scored on the basis of observed changes in their systolic blood pressure and heart rate. This scoring system was devised by Turner *et. al* and is reproduced here with his permission.

The method used to compute the scores summarised in Table 4.1 and Figure 4.1 [63] can be found in Appendix 3.

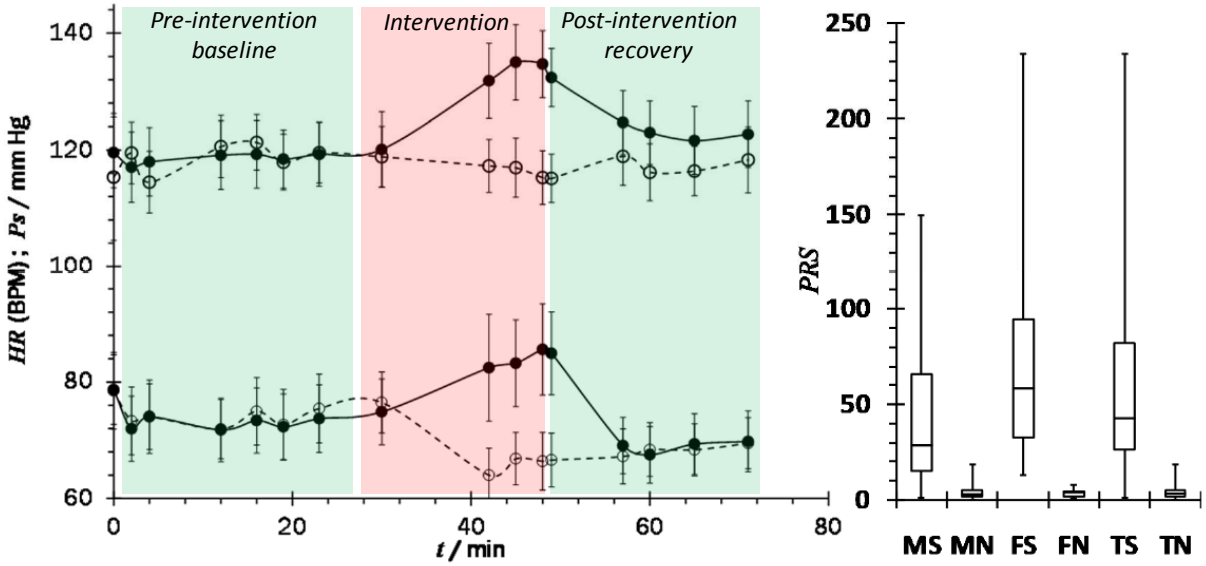


Figure 4.1 Heart rate and blood pressure data depicting evidence of a physiological response to induced psychological stress. Left, Average responses for systolic blood pressure (top) and heart rate (bottom) over the 70 minute PASAT (solid line) and relaxing music (dashed line) interventions. 0-23 min pre intervention baseline, 24-48 min intervention, 49-72 min post intervention recovery period. The error bars designate the 95% confidence limit on the mean. Right, box-whisker summaries of the physiological stress response (PSR) scores classified by male stressed and neutral interventions (MS and MN) and female stressed and neutral (FS and FN) and the total group stressed and neutral results (TS and TN). Figure reproduced from reference 62 with the author's permission.

The PSR sought to emphasise the cardiovascular responses to the intervention and relate them to the baseline measurements. The scores were framed to give a higher score to participants exhibiting the largest changes in systolic blood pressure and heart rate during the intervention. The PSR scores are summarized in Table 4.1 where PSR_P is the score for the PASAT intervention and PSR_N the score for the neutral intervention. ΔPSR is the neutral score subtracted from the PASAT score and for 14 out of the 15 participants ΔPSR is positive. This indicates a greater physiological response to the PASAT than the neutral intervention for these participants, consistent with the individuals experiencing stress during the PASAT intervention period.

Table 4.1 Physiological response scores to the PASAT (PSR_P) neutral (PSR_N)interventions, computed based on cardiovascular responses and reproduced from reference 63.

ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
PSR_P	0.9	30.3	12.9	56.7	26.1	74.2	87.6	84.5	19.7	149	34	233	40.8	27.5	72.6
PSR_N	5.3	1.6	7.4	1.9	2	3.2	15.5	5	4.2	18.6	7.7	2.7	1.2	4.5	1.4
ΔPSR	-4.4	28.7	5.5	54.8	24.1	15.5	72.1	79.5	71	130	26.3	230	39.6	23	71.2

4.4.3. Chromatographic data

Gas chromatographic-mass spectrometric (GC-MS) data was processed following the workflow described in Section 3.5 to produce a data-matrix. A total of 5 samples were lost due to a combination of failures in instrumentation or storage and, due to the randomized cross-over study design, this led to the exclusion of the other intervention for those participants. The final data set contained the data from 15 participants. VOCs were excluded from the matrix if they were found to be present in field blanks or were found in less than 30% of observations. This resulted in a skin VOC matrix of 30 observations (15 participants) and 40 variables (VOCs).
Figure 4.2 shows example chromatographic data for a single participant that has been aligned by retention index and undergone dynamic background compensation.

Initially partial least squares discriminant analysis (PLS-DA) was applied to the pareto scaled matrix and an S-plot generated. This S-plot, shown in Figure 4.3, indicated 4 candidate variables with significant contributions to differences between the profiles from the two interventions. Subsequent unsupervised principle component analysis (PCA) of these four variables enabled evaluation of the separation between the two interventions based on those 4 variables. The principle components identified exhibited 57% (PC1) and 27% (PC2) of the total explained variance of the data set. It was not possible at this stage to comment on the sensitivity and selectivity of the PCA as there seems to be some ambiguity over the clustering as shown in Figure 4.4; 87% of relaxed observations appear to sit within one cluster, whilst 87% of stressed observations sit in another. 3 observations, 2 relaxed and 2 stressed are located in a central region and it is unclear whether they belong to either of the existing clusters or form their own (Figure 4.4). This will be explored in more detail later in this Chapter.

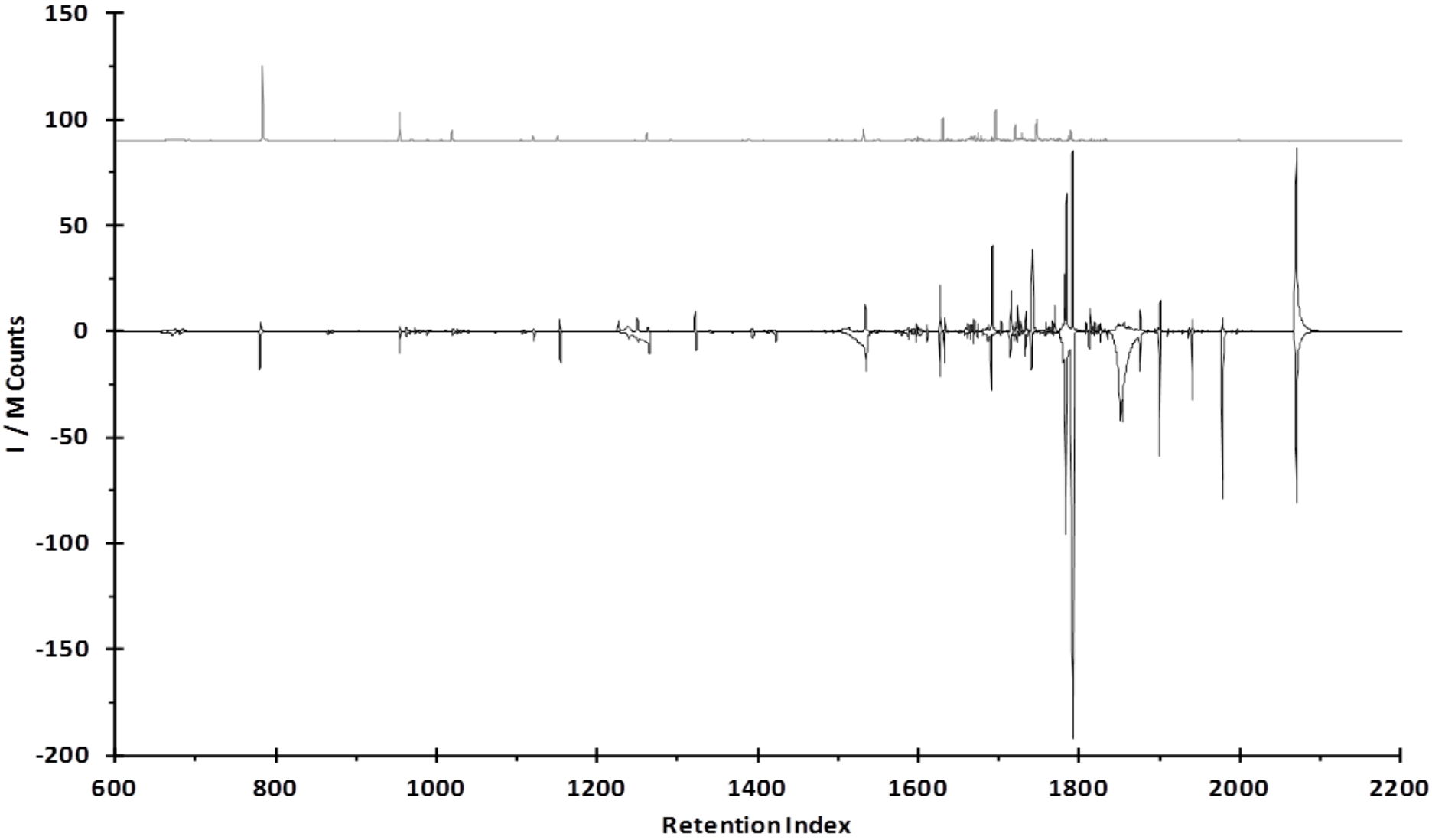


Figure 4.2 Example dynamic background compensated, retention index aligned skin TIC chromatograms from a female participant after the neutral intervention (top) and the PASAT (inverted), and a field blank (offset, grey).

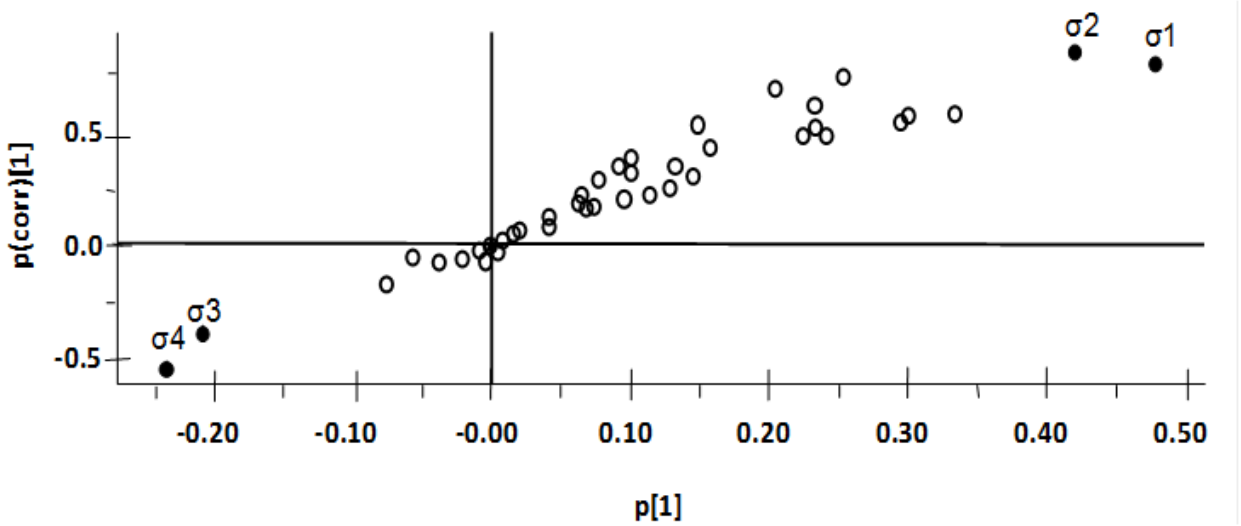


Figure 4.3 The loadings S-plot generated from the PLS-DA model for the metabolomic comparison between the PASAT and the neutral intervention skin VOC samples. The four variables highlighted (solid dots) at the extremes of the S-plot indicate potential markers sensitive to the PASAT intervention.

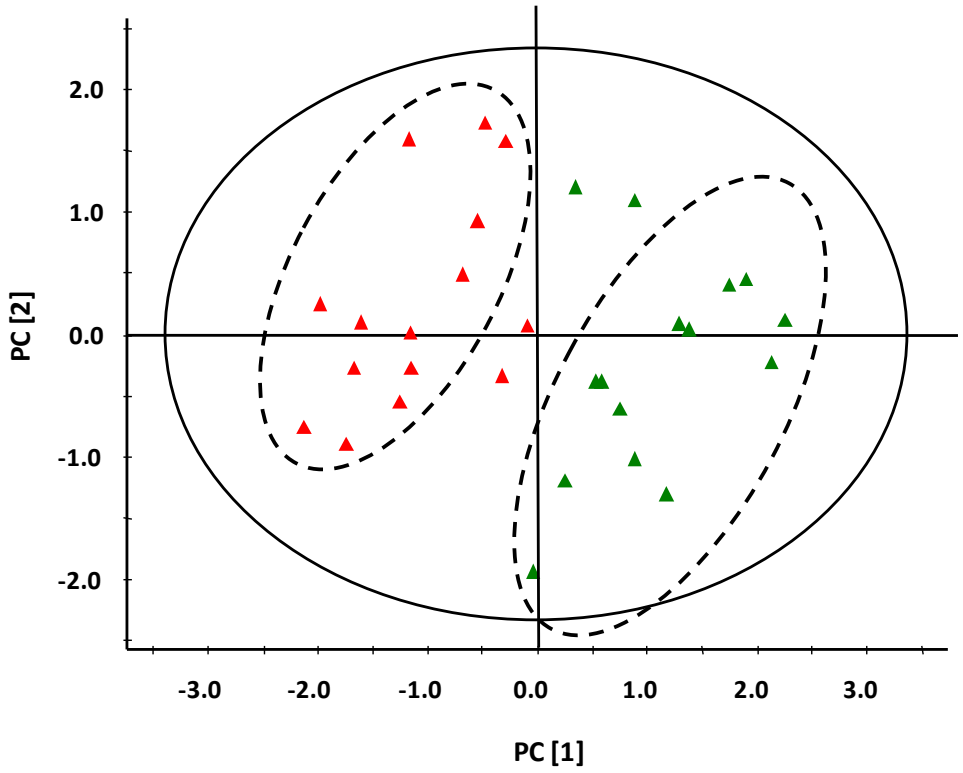


Figure 4.4 Unsupervised principle component analysis (PCA) of four potentially stress sensitive compounds identified from PLS-DA of skin VOC samples from PASAT and neutral interventions. Red markers denote the stressed condition and green the relaxed, 87% of stressed observations lie in one cluster, 87% of relaxed in another, 4 observations are unassigned.

Box-whisker plots were constructed from peak area responses for σ1 – σ4 in the stressed and relaxed states. The up-regulation of σ1 and σ2 in the stressed state and σ3 and σ4 in the relaxed state is clearly demonstrated in Figure 4.5.

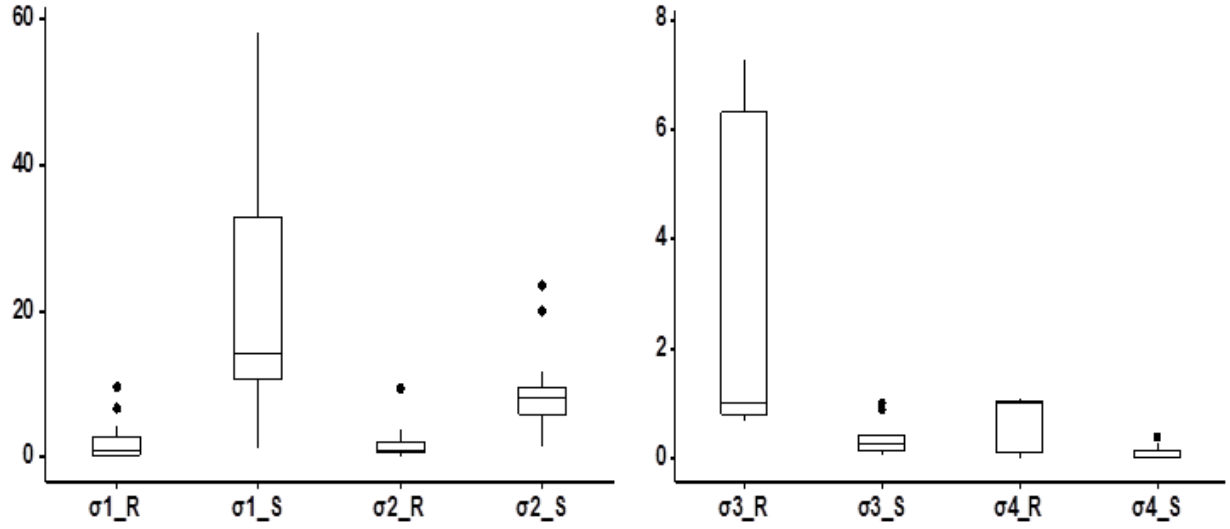


Figure 4.5 Box and whisker plots for the 4 stress sensitive skin VOC's identified by PLS-DA; the annotation _R denotes responses during the relaxed state and _S the stressed. Peak areas have been normalised so that the median of the relaxed phase is equal to 1, outliers are represented by the solid circles.

Receiver-operator characteristic (ROC) curves were constructed for the four candidate stress sensitive VOCs to determine the accuracy with which their responses predict the stressed state. Area under the curve (AUC) values were calculated from the ROC curves shown in Figure 4.6. AUC for σ1 and σ2 for predicting a stressed state were 0.813 and 0.852 respectively indicating that the level of these compounds is a good test for stress, AUC values for σ3 and σ4 for predicting a stressed state were 0.102 and 0.071 respectively indicating that these would be worthless for predicting a stressed state. For the prediction of an unstressed state σ3 and σ4 gave AUC values of 0.898 and 0.929 respectively again indicating a good test for the unstressed state.

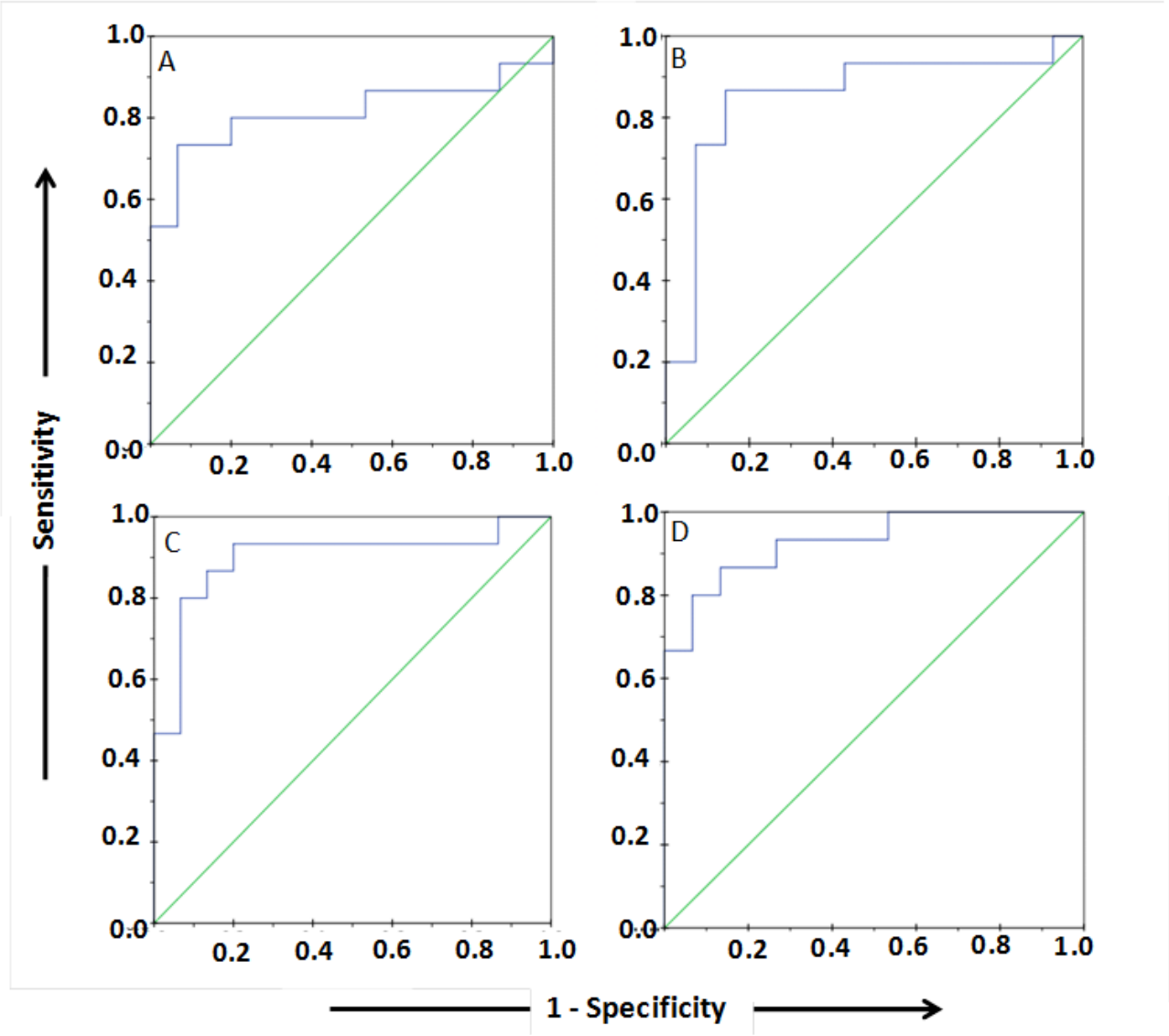


Figure 4.6 Receiver – operator characteristic curves for 4 candidate PASAT sensitive skin VOCs. A and B show curves for up-regulated compounds α1 (A) and α2 (B) predicting a stressed state. C and D show curves for down-regulated α3 (C) and α4 predicting an un-stressed state.

The candidate markers were tentatively identified based on spectral matching between their deconvolved mass spectra and the NIST mass spectral database (NIST 11) and a satisfactory match between the experimentally determined and published theoretical Kováts retention index. The criteria for these matches are detailed in Section 3.5.4.

Table 4.2 lists the library entry for each candidate marker, as described in Section 3.5.4 and the tentative identification. The deconvolved mass spectrum for each peak is listed as follows:

m/z of the base peak (Intensity normalized to 999), *m/z* of second most intense spectral peak (Intensity normalized relative to the base peak of 999),.....

The quality of the spectral match with the NIST mass spectral database is generated by NIST 11 as two scores: a forward (F) and a reverse (R) match. The 'forward' match score is the match factor for a direct comparison between the unknown spectrum and the library spectrum. The 'reverse' match score is the match factor for the unknown and the library spectrum ignoring and peaks in the unknown spectrum that are not present in the library spectrum. Both scores are calculated based on a modified cosine of the angle between the spectra [174], a perfect match returns a maximum score of 999, a result with no peaks in common returns a score of 0. Generally a score of >900 is an excellent match, 800 – 900 a good match and 700 – 800 a fair match.

Computation of the observed retention index value (O) is described in detail in Section 3.5.3, in Table 4.2 the expected retention index value (E) is the estimated Kováts retention index for the library match generated by NIST.

As yet it has not been possible to confirm these assignments by comparison of retention index and mass spectra with pure standards.

Table 4.2 **Tentative identification of PASAT sensitive skin compound σ1-σ4, including deconvolved mass spectra of the unknowns, forward (F) and reverse (R) NIST spectral match factor and observed (O) and expected (E) retention indices for the unknown and library match respectively.**

Ref	Name / Library Entry	Deconvolved Mass Spectra	NIST Match (F/R)	Retention Index (O/E)
σ1	Benzoic Acid Sk-1262-105-122-77-51	105 (999), 122 (857), 77 (700), 51 (357)	866 / 924	1262 / 1271
σ2	n Decanoic Acid Sk-1380-60-73-41-43	60 (999), 73 (920), 41 (508), 43 (480)	764 / 782	1380 / 1385
σ3	p Xylene Sk-862-91-106-105-77	91 (999), 106 (600), 105 (260), 77 (125)	914 / 914	862 / 872
σ4	3 – Carene Sk-995-93-91-77-79	93 (999), 91 (560), 77 (320), 79 (298)	872 / 879	995/ 1010

Example responses for these four compounds from a female participant (participant 4) are shown in Figure 4.7.

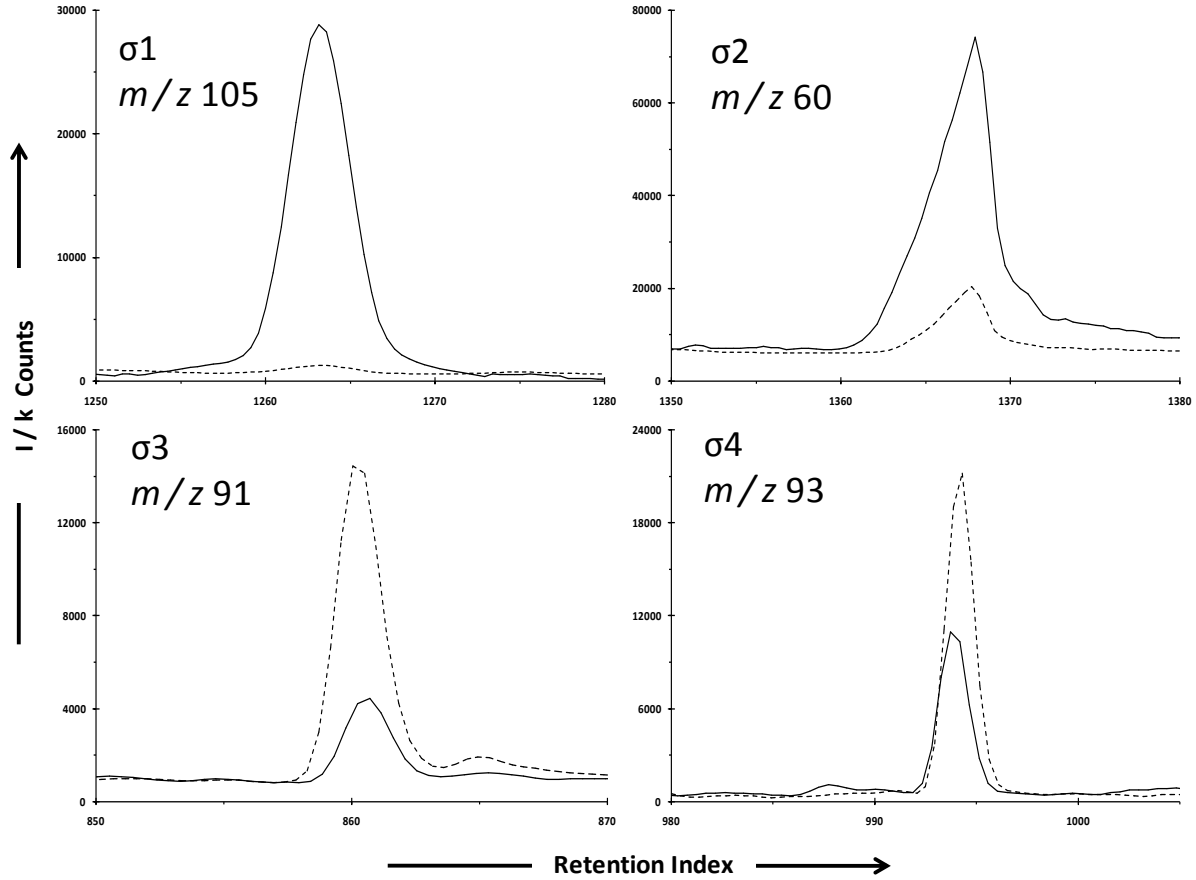


Figure 4.7 Base peak extracted ion chromatograms (EIC) from a single participant for the candidate stress sensitive skin VOC σ1 (*m/z* 105), σ2 (*m/z* 60), σ3 (*m/z* 91) and σ4 (*m/z* 93) . EIC from the PASAT session (solid line) are overlaid with those from the neutral session (dashed line) for each candidate.

The scores plot produced from unsupervised principle component analysis showed some ambiguity in the clustering with 4 observations lying between the two distinct clusters, two from the stressed and two from the relaxed states. In an effort to better classify these observations as belonging to one group or the other a dendrogram was constructed. Distances between clusters were calculated by Ward's method which joins groups so that the smallest possible change in a given measure of heterogeneity is achieved. In this way the most similar clusters are joined minimising the within cluster variance with each linkage [153].

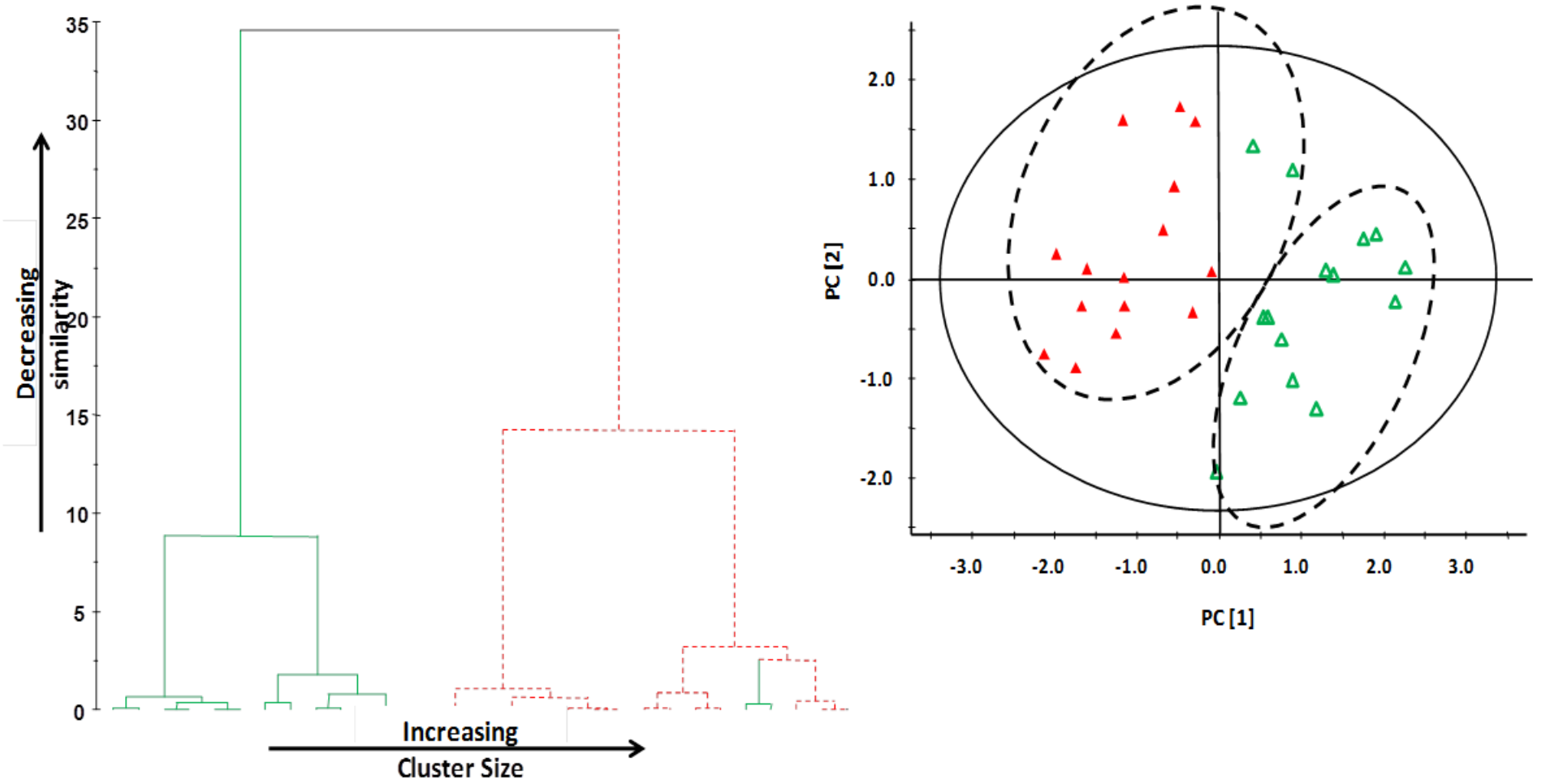


Figure 4.8 A dendrogram constructed from unsupervised PCA of 4 candidate stress sensitive skin VOCs to determine the degree of similarity between stressed and unstressed states. Left: Dendrogram constructed from scores and loading from unsupervised PCA showing good clustering for the relaxed and stressed states. The solid green bars indicate relaxed observations and the dashed red stressed; the length of the vertical bars indicates similarity with longer bars denoting a reduction in similarity. Two relaxed observations exhibit more similarity with the stressed than the other relaxed states and can be found to the right of the dendrogram. Right: unsupervised PCA from Figure 4.4 redrawn to show the 4 previously unassigned observations now associated with the 'stressed' cluster based on information from the dendrogram.

The dendrogram constructed from PCA of the four candidate markers from this study shows good clustering for the neutral and stressed states, with two participants from the neutral condition sitting within the stressed cluster (Figure 4.8). As observed in Figure 4.4, the scores plot shows two stressed observations, participants 3 and 5, and two relaxed observations, participants 3 and 6, that are not clearly associated with one cluster or the other. The hierarchical clustering depicted in the dendrogram indicates that these 4 observations should be included in the 'stressed' cluster, the implication being that participants 3 and 5 were not relaxed during the neutral intervention.

4.4.4. Comparison with breath

An in-depth discussion of the results from the breath samples [62] does not form part of this thesis. Conceptually the prospect of injury-free, non-invasive breath sampling for metabolic investigation is attractive. However isolation of the low-concentration endogenous VOCs in exhaled air requires representative sampling of the alveolar, or end-tidal, portion of exhaled breath and the challenges associated with this were discussed in Section 1.1.3. The principle challenge with collecting breath samples when compared with skin samples is the additional level of complexity in the sampling procedure, and the necessity for specialised equipment to ensure a reproducible and representative end-tidal breath fraction is sampled. In this study an adaptive breath sampler, described in detail elsewhere [63] was used to achieve this.

The adaptive breath sampler is specialist instrumentation requiring accompanying utilities such as power and a supply of clean compressed air. The operator needs to be a trained scientist, and as such despite many advantages over blood and urine based approaches, does not completely fulfill the criteria for the ideal non-invasive sampler, Section 1.1.3. Breath sampling is technically difficult and the sampling itself requires trained personnel and is not self-administrable.

The Skin Patch was well received by participants and no adverse effects were reported. It is unobtrusive, simple to use, self administrable depending on sampling site, does not restrict the participant to one location during sampling, requires no external power or other utilities and is easily transported and stored allowing for batch sampling in–field and off-line analysis. In many regards the Skin Patch fulfils the criteria for the ideal non-invasive diagnostic tool. The question remains as to whether any biologically significant information can be recovered from skin VOC profiles.

Visual inspection of skin and breath chromatograms from the same participant in this study reveals approximately 100-150 more compounds to have been recovered from the skin; this becomes particularly apparent for higher molecular weight compounds which are less likely to

be found in exhaled breath than on the skin surface. Figure 4.9 shows example skin and breath samples from one participant in this study which have been aligned by retention index, it is important to note that these samples were analysed on different analytical systems and so a comparison of absolute response is not possible.

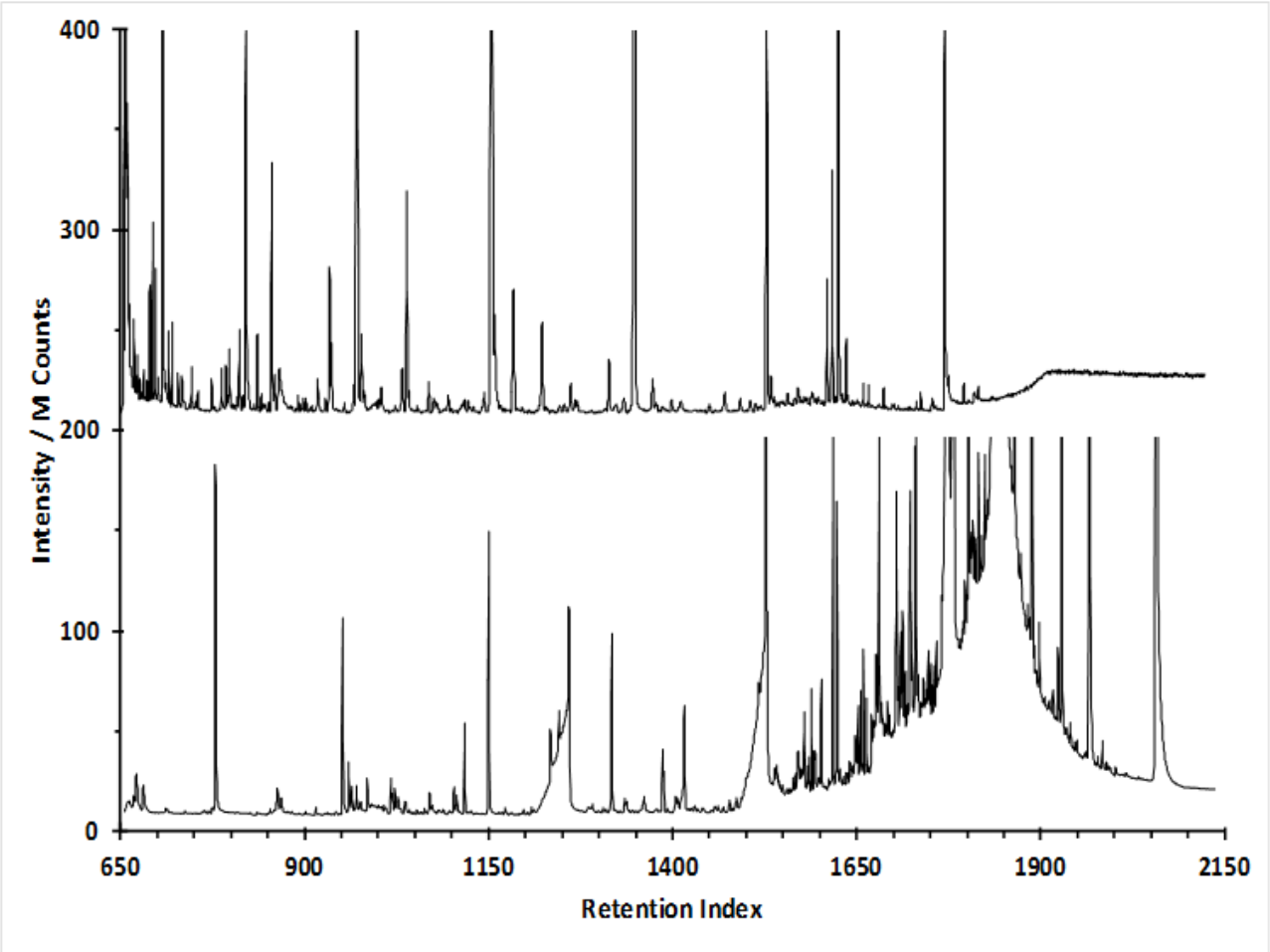


Figure 4.9 Example skin and breath VOC TIC chromatograms from a single participant. (Top) Example breath VOC profile shown at 50 fold magnification and (Bottom) example skin VOC profile from the same participant shown at 10 fold magnification, both chromatograms are shown with a maximum intensity of 200 M Counts. The shape of the profiles demonstrates the affinity of the PDMS Skin Patch for higher molecular weight semi-volatile compounds whilst the breath profile is most rich towards the more volatile end of the chromatogram.

A suite of candidate stress-sensitive biomarkers was generated from the breath study following an analogous work flow to that described above for the skin data. This facilitated easy interrogation of the skin VOC profiles to determine whether the breath markers were present in the emanations from the skin and, if so, did they exhibit any changes associated with the stress intervention? Tables 4.3 and 4.4 detail the proposed stress sensitive markers and their retention index values in both skin and breath sample compared to the theoretical value (NIST 11).

Table 4.3 Proposed stress sensitive *breath* markers, from reference 62, and their experimentally determined retention index values in skin and breath samples compared with theoretical retention indices from NIST.

Breath Marker	BRI	SkRI	NIST RI
Indole	1286	Not Found	1276
2-hydroxy – 1 - phenylethanone	1063	1075	1272
2-methylpentadecane	1527	1483	1548
Unknown terpene	951	995*	N/A
Benzaldehyde	976	955	982
2-ethylhexan-1-ol	1019	993	995

* Gives good spectral match with σ4, proposed 3-Carene Figure 4.10

Table 4.4 Proposed stress sensitive *skin* markers and their experimentally determined retention index values in skin and breath samples compared with theoretical retention indices from NIST.

Skin Marker	BRI	SkRI	NIST RI
Benzoic Acid	1195	1262	1271
n-Decanoic Acid	Not Found	1380	1371
p-Xylene	903	862	872
3-Carene	951	995	1010

None of the compounds identified as changing in response to the PASAT in the skin study were found to change in the breath data. Two PASAT sensitive breath compounds, 2-ethylhexan-1-ol and the unknown terpene, Figure 4.10, now tentatively identified as 3-carene, were also found to change in the skin samples.

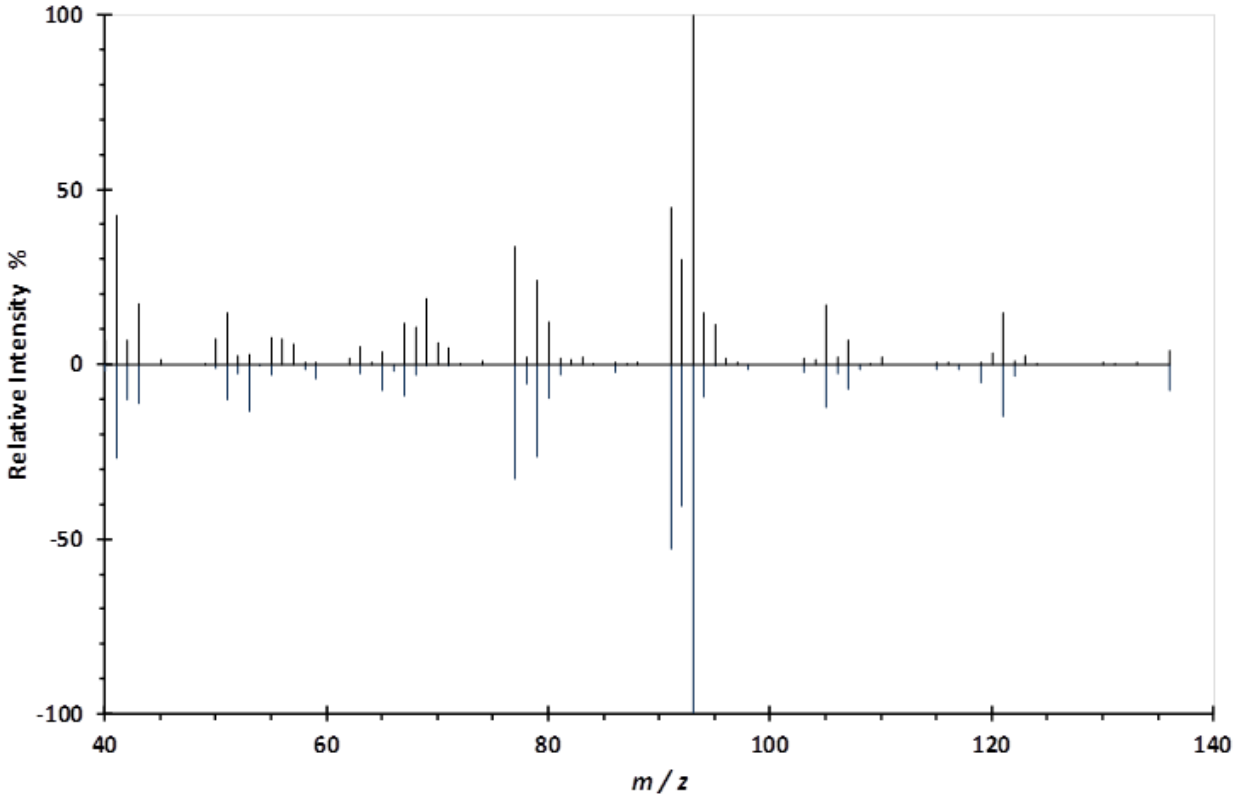


Figure 4.10 Mass spectra of an unidentified terpene compound found to be down-regulated in both breath and skin VOC samples after the PASAT intervention. Top: mass spectrum for the unidetntified terpene with a etention index of 951 from the breath study [62]. Inverted: σ4 with RI 995, tentatively identified as 3-carene, from the skin study.

The breath samples from this study were analysed using a different TD-GC-MS instrument fitted with a 30 m column, of the same stationary phase, film thickness and internal diameter, as opposed to the 60 m column used in the skin analysis. Using the workflow described in Section 3.5 it was possible to compare data generated from these studies using retention index alignment where a maximum drift in retention index between compounds on the two systems was 67 RI units. The overall duration for this study, for analysis of skin and breath samples, was 19 weeks during which instrument maintenance was necessary on several occasions and the retention indexing protocol described in Section 3.5.3 was needed to correct for changes in retention time.

The biological origin of the candidate stress sensitive VOCs observed from skin and breath samples is not well defined and their presence in the VOC profiles may result from a combination of endogenous and exogenous origins. It is not possible to postulate metabolic pathways and roles for these compounds as part of a stress sensitive response in the human body.

It is interesting to note that only two VOCs were observed as potentially stress sensitive in both skin and breath. 2-ethyl-hexan-1-ol has been reported as a potential marker for lung cancer in human breath and was found to decrease in response to psychological stress in breath [62] [175]

, whilst in skin profiles it was found to increase, whether this phenomenon is due to an increase in blood flow under the skin surface with the increase in cardiovascular activity accompanying stress leading to faster clearance from the blood, or a change in metabolic processes as a part of the 'fight or flight' response or due to something else entirely is unclear and not the focus of this study. The unidentified terpene from the breath study shows good spectral matching with σ4 from the skin study and the retention index values are only 17 units outside the tolerance set in this study, giving a suggestion that these may be the same compound; however without analysing a pure standard it is not possible to confirm this suspicion. What is evident, however, is that for both compartments a volatile terpene compound was shown to be down–regulated as a response to the stress intervention. The origin of terpenes in breath and skin profiles is not well defined and may not be endogenous. Terpenes occur naturally in foodstuffs [176], yet the data from this pilot study would indicate that they are perhaps involved in the metabolic processes associated with the body's response to psychological stress.

Benzoic acid has been reported as a metabolite of both benzaldehyde and toluene and studies evaluating the safety of benzaldehyde use in cosmetic products list benzoic acid as its primary metabolite [177]. Boehlein et al reported the oxidation of benzyl alcohol to benzoic acid in skin by alcohol dehydrogenase with benzaldehye as the presumed intermediate [178] while Turner *et al* reported a down regulation of benzaldehye in breath with the PASAT stress intervention [62], suggesting that oxidative metabolic pathways may be increasing in activity with stress. Occurring commonly in food stuffs, benzaldehyde is readily absorbed through the skin and lungs and, along with its metabolites, distributes to all well-perfused organs and does not bioaccumulate in any one tissue type. Its primary metabolite is benzoic acid, which conjugates with glycine or glucaronic acid and is excreted in urine. Oxidation of benzyl alcohol and benzaldehyde appear to be the most commonly documented source of benzoic acid in urine, blood and on skin [177-179]. Benzoic acid has also been reported as a biomarker for asthma and lung cancer in exhaled breath [180] [181].

A wide range of short (C2-C5) and medium (C6-C11) chain volatile fatty acids are frequently reported in apocrine sweat as a source of malodour. These were commonly understood to carried to the skin surface as glutamine conjugates and released by bacterial action [182]. A major route to short and medium chain volatile fatty acids in axillary secretions was shown to be partial catabolism of methyl branched longer chain fatty acids by the Corynebacterium bacteria; resident at highest levels in the axilla and on the forehead [183] [184]. Structurally unusual fatty acids such as these methyl branched analogues are reported to originate from triacylgylcerol component of sebum, and from apocrine sweat, and are released by bacterial

lipases. Up-regulation of medium chain fatty acids such as n-decancoic acid reported in this study may be the result of an increase in apocrine sweat, elevated bacterial action with increased skin temperature or as the end product of a longer metabolic change increasing the secretion of methyl branched fatty acids precursors to the skin surface [182] [183]. Methylated hydrocarbons have been reported as markers for oxidative stress and numerous compounds in this class have been proposed as biomarkers for cancers [65] [185]. There is no evidence linking these to the up-regulation of skin surface volatile fatty acids, in particular decanoic acid, observed in this study. This once again indicates that links between psychological stress and a potential increase in oxidative metabolic pathways warrants more in-depth investigation.

The source of xylene on skin is likely to be at least partly exogenous; exposure is generally via household cleaning products, vehicle exhausts and cigarette smoke [186]. Xylenes are highly lipophillic so are absorbed readily through the skin and distribute quickly throughout the body [187], p-xylene has also been reported as a biomarker for lung cancer in exhaled breath [181].

4.5. CONCLUSIONS

This pilot study set out to evaluate the use of the Skin Patch sampler for metabolomic studies. Observations during sampling showed the Skin Patch to be comfortable for participants and simple to use. The Skin Patch was also easy to combine with other sampling techniques and allowed for short term storage and off-line analysis of skin samples.

This pilot study has shown that the VOCs recoverable from human skin are wide ranging both in the class and concentration of compounds. Ubiquitous siloxane compounds may be exploited as internal standards for retention indexing, facilitating successful alignment of chromatograms analysed over several months, while strict control of the analytical instrument performance with regular external quality control standards could enable semi-quantitative comparisons between peak area responses. Evaluation of the retention indexing strategy revealed greater errors in retention index accuracy for those compounds eluting during the retention time periods when the GC oven temperature ramp rate (GC programme 2) was changing; this was observed both between skin and breath samples on the two analytical systems and between skin samples run on the same instrument. As a result subsequent studies employed a linear temperature programme to improve accuracy in retention index alignment with no loss in chromatographic quality (GC programme 3).

While a small number of samples were excluded due to failures in storage or instrumentation, another set were excluded based on visual inspection of the chromatograms; significant drops in baseline intensity or skewing of mass spectra, so that the response of higher *m/z* values was significantly reduced, was observed after particularly high concentration samples and

attributed to 'dirtying' of the ion source. If this was observed a QC standard was analysed and the decision taken whether to proceed or pause analysis to perform maintenance based on the responses across the QC range, this inevitably caused the suspect sample data to be excluded from data processing. The desirability of introducing an internal standard to reduce the subjective nature of this screening process became apparent during this study and will be explored in Chapter 6.

Stress changes in the VOC profile recovered from the skin of healthy volunteers were observed in this pilot study. Four compounds, present in >30% of observations, were found to contribute significantly to these changes and a tentative correlation with physiological response to induced stress was observed.

Two compounds were found to increase with stress and these have been tentatively identified as benzoic acid and *n*-decancoic acid. The biological origin of benzoic acid has yet to be completely described. Literature evidence suggests its presence on skin to be primarily due to oxidation of benyl alcohol and benzaldhyde. The biological origin of the two down-regulated VOCs, tentatively idenitifed as p-xylene and 3-Carene, is as yet unclear.

Comparison of these results with breath samples generated from the same study but analysed on a different TD-GC-MS system has allowed evaluation of the workflow described in Chapter 3 and demonstrated the ease with which data from different studies, systems or even laboratories can be compared when this protocol is adhered to. This study has also highlighted the weaknesses with the retention index alignment approach for GC temperature programmes with more than one ramp rate and this was addressed for subsequent studies.

Whilst the majority of the most stress sensitive skin compounds were present in breath, and vice versa, only two compounds were found to change significantly with stress in both compartments. The implications of this observation could be wide-ranging and varied and it is not the focus of this study to compare the chemistries associated with the two sample types. It is perhaps a good point to highlight that, whilst the compounds identified as changing in response to stress were shown to be the most statistically significant in these studies, it does not necessarily follow that they are the most biologically significant and there may be a sub-set of data showing better correlations between breath and skin.

The Skin Patch has been demonstrated to perform reliably in a pilot metabolomic study and generate data allowing classification of skin profiles from stressed and relaxed individuals. The ease of use, transport and storage of the Skin Patch has distinct advantages over breath sampling for larger or more remote studies and it fulfils more of the criteria described in Section 1.1.3 for the ideal non-invasive sampler. The potential for confounding factors with the

skin sampling will always be much higher than with breath samples, when such samples include only the end-tidal portion of an exhaled breath.

The possible correlations observed with down-regulation of benzaldehyde in breath and up-regulation of its well known oxidation product benzoic acid in skin, as well as the down-regulation of terpene compounds in both compartments, raise interesting questions about the transport of biological VOCs into and out of skin and breath. These merit further characterisation studies of their own. At this stage it may be advantageous to operate with the working hypothesis that skin and breath profiles are complementary, with the possibility that markers identified from breath may undergo biotransformations before reaching the skin surface that could be applied to clinical screening scenarios in a more targeted approach – enabling all the advantages of the Skin Patch to be employed in the larger scale studies.

The continuation of this work into a larger panel study and investigation of other stressors will build on the initial observations made here and improve understanding of skin VOC profiling as a potentially useful, non-invasive measurement of human function.

5 THE EFFECT OF INDUCED PHYSIOLOGICAL STRESS ON VOC PROFILES OF YOUNG ADULTS: COMPARING SKIN AND SALIVA SAMPLING IN A PILOT STUDY

5.1. INTRODUCTION

Extending the application of a 'stressor' as a surrogate for disease state, this study employed a physical stress task which enabled a different set of physical and biochemical processes to be evaluated, and for the first time to include the saliva sampling protocol developed in Section 3 in a pilot metabolomic study. The primary research questions were:

- Was it possible to observe a measurable change in skin and saliva VOC profiles attributable to physical stress?
- Which of the two approaches might yield the "best" approach to physical stress measurements and, by implication, other perturbations of metabolism?
- Would it be possible to discern any metabolic links between the two profiles?
- Would there be any benefit in combining the data sets?

5.2. SAMPLING AND ANALYSIS CAMPAIGN

This study was devised and carried out in collaboration with Matthew Turner, Pareen Patel and Aditya Malkar. The workflow incorporated exhaled breath for VOC analysis, drooled saliva, whole blood and urine for elemental and metabolite analysis as well as skin and saliva for VOC analysis. A detailed study timeline may be found in Appendix 4. The results of the studies based on the additional sample sets are discussed elsewhere [46], and are beyond the scope of this thesis. The study was carried out in collaboration with Prof. Ronald Maughan and Dr Phillip Watson of the School of Sport, Health and Exercise Sciences at Loughborough University.

The sampling campaign ran over 5 consecutive days in an environmentally controlled chamber, in the School of Sport Health and Exercise Science at Loughborough University. Ten healthy non-smoking adult males aged 18-35 volunteered to participate and attend a single 2 hr long study visit. The study session was run over four consecutive phases, each 30 minutes in duration:

- Phase A – Baseline measurements, pre-exercise phase. (0 – 30 min)
- Phase B – Exercise on cyclometer with a workload of 2 W/kg. (30 – 60 min)
- Phase C – Recovery phase 1, post exercise rest phase. (60 – 90 min)
- Phase D – Recovery phase 2, post exercise rest phase. (90 – 120 min)

Baseline measurements and samples were taken at the start of the session (Phase A) before physical stress was induced by peddling on a cyclometer at a workload of 2 watts/kg of body mass for 30 min (Phase B). At the end of the 30 min exercise intervention sampling and monitoring took place over two consecutive 30 min recovery phases, during which the participants rested as their metabolism returned to their pre-intervention baseline physical state (Phases C and D).

VOC samples were obtained following the protocols described in Sections 3.2 and 3.3; skin VOC samples were taken during all four phases and saliva during phases A, C and D. Each participant's heart rate was monitored throughout the study visit and recorded at 10 minute intervals during rest phases and 5 minute intervals during the exercise phase.

Saliva VOC samples were stored at 4 °C and analysed within 24 hours of sampling while skin-patches were stored at -80 °C for a maximum of 21 days before analysis. The analytical protocol is described in Section 3.4, GC programme 3 was employed for this study.

5.3. RESULTS AND DISCUSSION

The skin and saliva sampling protocols were easy to implement and work into a complicated study timeline and well tolerated by volunteers. The simplicity of these sampling techniques was highlighted when deployed in the field alongside the others sample collection techniques; breath sampling required transportation of an air compressor, blood sampling required a trained technician to be available and further processing before storage. Urine and drooled saliva samples also required immediate processing prior to storage and transport. One researcher was therefore dedicated to processing blood, urine and drooled saliva samples throughout each study visit totaling 20 hours work in a 10 participant pilot study. Skin & saliva VOC samples, however, simply required immediate transfer to thermal desorption tubes and temporary storage on dry ice until return to the laboratory within 6 hours.

5 biological compartments were sampled in this study and, despite the heavy sampling burden, a rigorously controlled study timeline enabled a unique set of samples to be obtained. Metabolomic analysis of the profiles from these samples will provide insight into the effect of physical stress on elements, VOCs, SVOCs and non-volatiles in skin, saliva, breath, urine and blood. The results from these will be combined to give a description of the metabolic changes associated with physical stress. The stitching and global analysis of the full sample suite is beyond the scope of this thesis. This chapter will focus on VOCs and SVOCs from skin and saliva samples.

5.3.1. The effect of induced physiological stress on VOC profiles from human skin

Upon visual inspection of the skin VOC profiles across the four phases the effect of the exercise intervention on the dominant features is evident. Figure 5.1 shows the raw TIC chromatograms from one participant for skin samples from Phases A – D. An increase in concentration of compounds eluting between 30 and 40 minutes is clearly visible from Phase A, the baseline phase, to Phase B, the exercise intervention. There is evidently a large amount of information obscured in the unresolved chromatography in this region and this will be discussed further in Chapter 6.

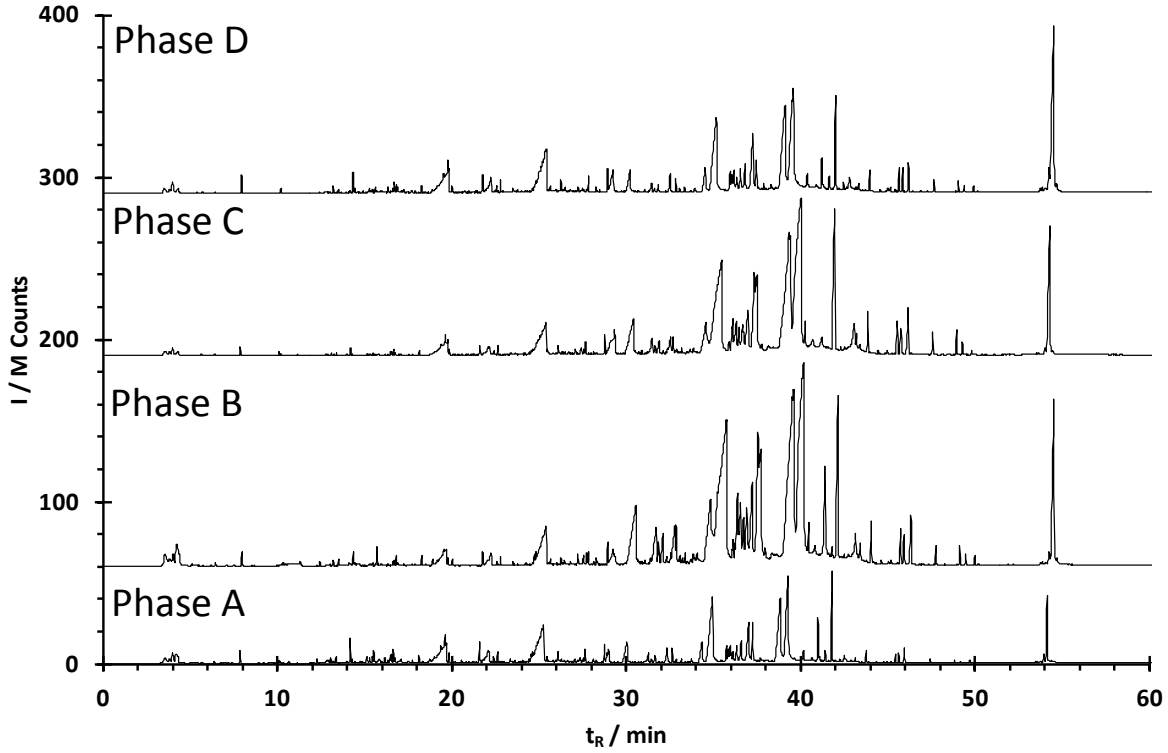


Figure 5.1 Unprocessed TIC chromatograms of skin samples from a single participant subject to a physical stress intervention. Phase A (bottom) is the pre-exercise baseline measurement, Phase B (offset at 60 M Counts) was sampled during exercise, Phase C (offset at 190 M Counts) is the first 30 minute recovery period after exercise and Phase D (offset at 290 M Counts) is the second 30 minute recovery period immediately following Phase C.

A pareto-scaled data matrix of 107 variables and 20 observations, derived using the workflow described in Section 3.5, populated with peak area responses from the 10 Phase A and 10 Phase B observations was subject to PLS-DA and the resultant S-Plot is shown in Figure 5.2. Eight variables from the extremes of the S-Plot were selected as candidates differentiating between participants in a baseline and a physically stressed state.

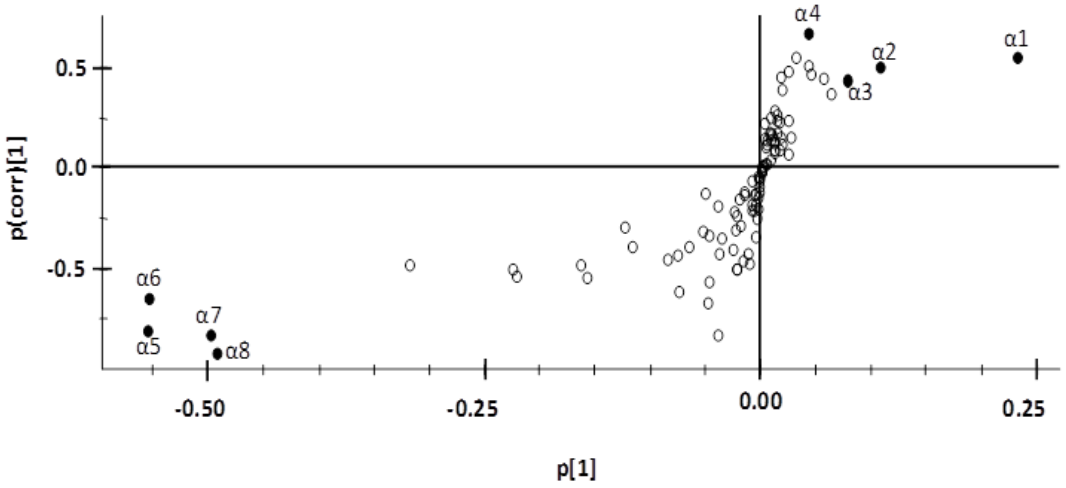


Figure 5.2 The loadings S-plot generated from the PLS-DA model for the metabolomic comparison between pre-exercise (Phase A) and during excerice (Phase B) skin VOC samples. The variables highlighted (solid dots) at the extremes of the S-plot indicate potential markers sensitive to the exercise intervention.

The candidate markers highlighted in Figure 5.2 were tentatively identified based on spectral matching between their deconvolved mass spectra and the NIST mass spectral database (NIST 11) and a satisfactory match between the experimentally determined and published theoretical Kováts retention index. The criteria for these matches are detailed in Section 3.5.4. Table 5.1 lists the library entry for each candidate marker, as described in Section 4.3.3 and the tentative identification.

Table 5.1 Tentative identification of exercise sensitive skin compounds α1-8, including deconvolved mass spectra of the unknowns, forward (F) and reverse (R) NIST spectral match factor and observed (O) and expected (E) retention indices for unknown and library match respectively.

Ref	Name / Library Entry	Deconvolved Mass Spectra	N (F/R)	RI (O/E)
α1	9-Octadecanal Sk-2009-55-69-67-54	55 (999), 69 (400), 67 (380), 54 (307)	770 / 823	2009 / 2007
α2	E-2-Nonen-1-ol Sk-1121-57-41-43-68	57 (999), 41 (669), 43 (550), 68 (230)	854 / 849	1121 / 1120
α3	2-Butanoylfuran Sk-1057-95-110-138-123	95 (999), 110 (560), 138 (100), 123 (45)	820 / 799	1057 / 1077
α4	Tridecanoic Acid Sk-1659-73-60-43-55	93 (999), 91 (560), 77 (320), 79 (298)	872 / 879	1659 / 1670
α5	n-Decanoic Acid Sk-1402-60-73-55-129	60 (999), 73 (900), 55 (400), 129 (350)	786 / 787	1402 / 1385
α6	Palmitoleic Acid Sk-1929-55-69-83-97	55 (999), 69 (810), 83 (620), 97 (480)	903 / 897	1929/ 1936
α7	2, 3-Dichlorobenzoic Acid Sk-1521-173-190-175-192	173 (999), 190 (810), 175 (635), 192 (500)	935 / 941	1521 / 1510
α8	Octanoic Acid Sk-1220-60-73-55-101	60 (999), 73 (630), 55 (375), 101 (240)	907 / 913	1220 / 1197

Unsupervised PCA was applied to a secondary matrix constructed of peak area responses for the eight variables (candidate compounds) in all four experimental phases (40 observations) the scatter plot generated is shown in Figure 5.3 and shows that these compounds differentiate between the skin VOC profiles recovered across the 4 experimental phases.

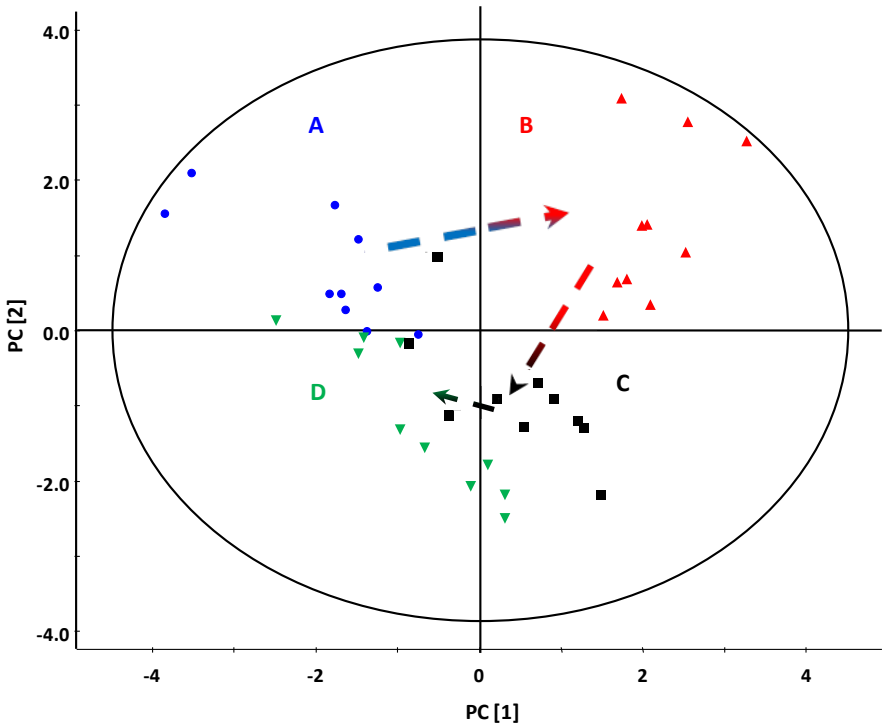


Figure 5.3 Unsupervised principle component analysis (PCA) of eight candidate exercise sensitive VOC selected from PLS-DA of skin VOC samples. Blue dots show the pre-exercise baseline measurements (Phase A), red triangles show the exercise phase (Phase B), black squares show the first recovery stage (Phase C) and inverted green triangles show the second recovery phase following on from Phase C (Phase D).

Principle components 1 and 2 identified here exhibit 38 and 28% respectively of the total explained variance of the data set across all 4 experimental phases. The perturbation of the skin VOC profile with physiological stress is evident as is the subsequent relaxation towards the baseline state with each recovery phase. Closer inspection of the raw peak area responses for each of the candidates reveals two behaviour types; one sub-set of candidates (subset A) exhibits a change with physical stress that tends to relax towards the baseline level by Phase D shown in Figure 5.4. The remaining candidates (subset B) also exhibit a change with stress, although they do not return to the resting state in the timeframe monitored in this study, shown in Figure 5.5.

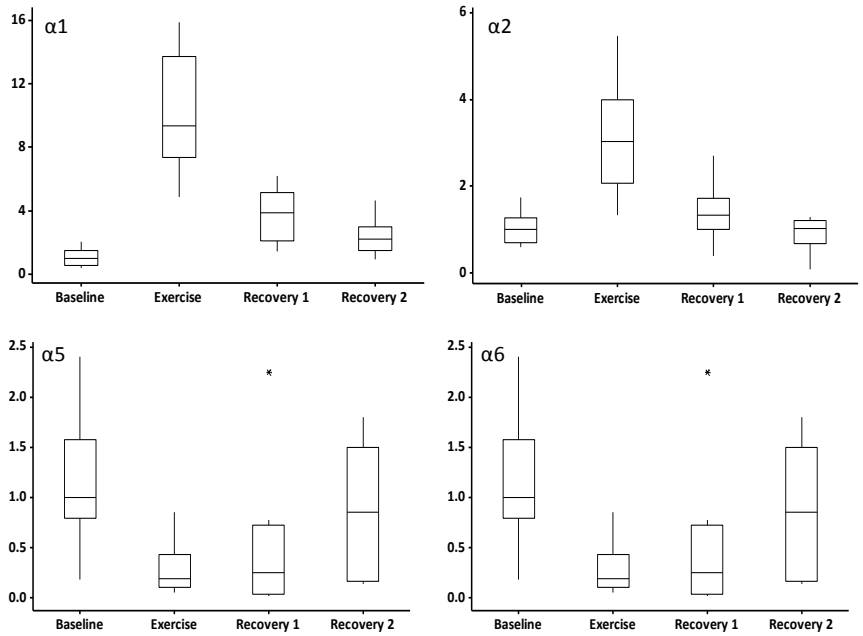


Figure 5.4 Box and whisker plots of peak areas for 4 of the execrise sensitive skin VOCs that relax towards the baseline response during the monitored recovery phases, C and D. Peak areas have been normalised so that the median of the baseline phase is equal to 1.

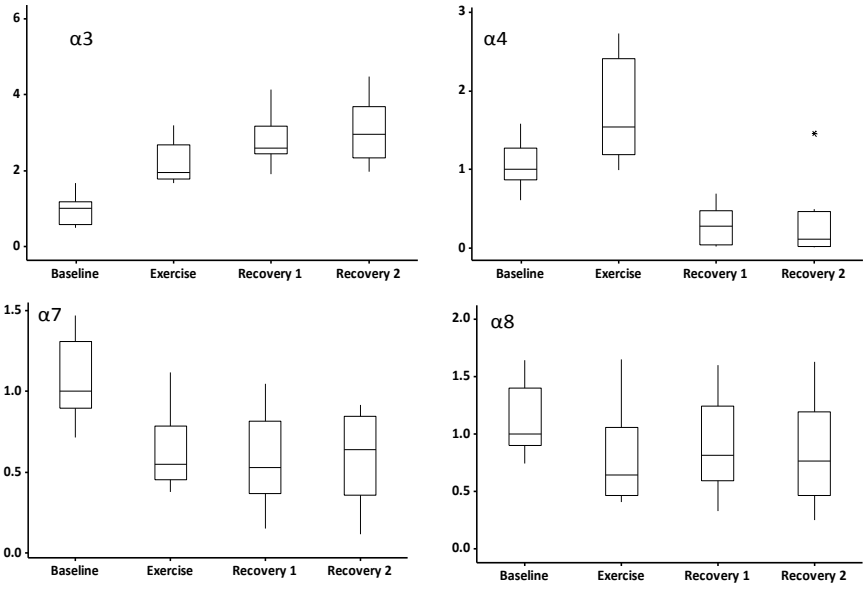


Figure 5.5 Box and whisker plots of peak areas for 4 of the exercise sensitive skin VOCs for whom baseline state is not recovered during recovery Phases C and D. Peak areas have been normalised so that the median of the baseline phase is equal to 1.

Exploration of the set of candidates that relax towards the baseline state indicates these may be sensitive to short term, rapid physiological changes associated with exercise, elevated heart rate for example. Figure 5.6 explores the correlation between two such compounds; α1 up-regulated with exercise and α6 down-regulated, and change in heart rate.

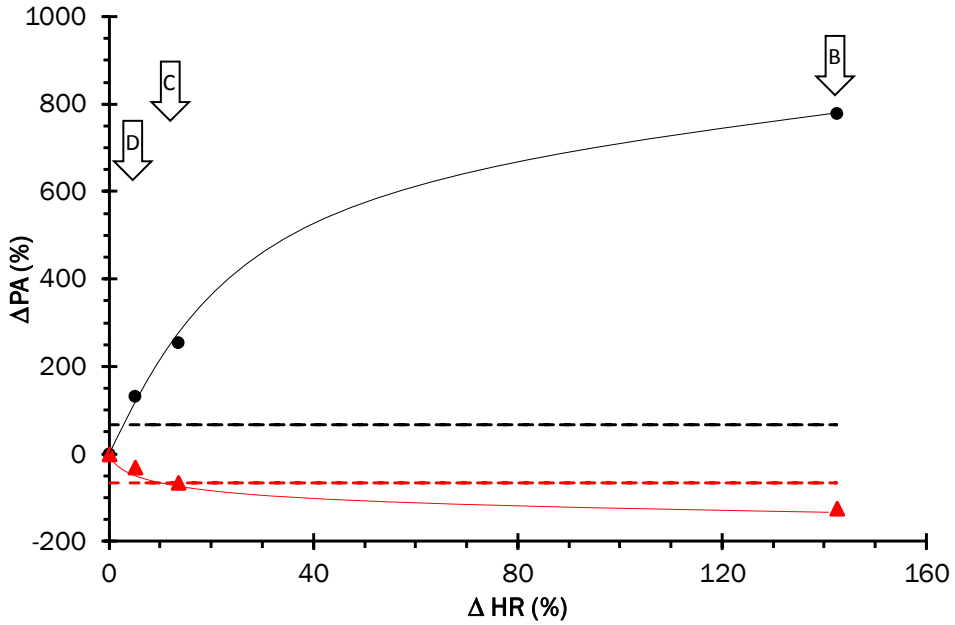


Figure 5.6 **The relationship between percentage change in peak area response and percentage change in heart rate for candidate exercise sensitive compounds α1 and α6. Up-regulated α1 (black) and down-regulated α6 (red) relationship with ΔHR, dashed lines represent upper 95% confidence limit on baseline responses.**

Figure 5.6 shows the correlation between global averages of percentage change in peak area and percentage change in heart rate over the 4 study phases. Excluding the exercise phase R^2 values for ΔHR (%) against ΔPA (%) ranged from 0.9726 to 0.9899 for subset a, indicating a degree of correlation, when phase B is included these graphs appears to plateau. The implications of these observations may be that these compounds are up- or down- regulated during exercise as the result of a faster heart rate; a combination of vasodilation and higher velocity blood flows under the skin surface serve to shorten the pathlength and increase concentration gradients between compounds in circulating blood and on the skin surface.

Not all of the compounds identified as changing between the rest and exercise phases can be explained by a higher heart rate and greater volume of blood passing under the skin surface; α3, 7 and 8 do not return towards the baseline level during the recovery phases monitored while α4 peaks during exercise then drops below the baseline level and remains there for the duration of the recovery phases. An equivalent plot to Figure 5.6 for these candidates shows no correlation between ΔPA and ΔHR. These changes may be indicative of a longer metabolic chain associated with the physical stress that takes longer than the hour monitored post exercise to recover; heart rate is not the only factor contributing to changes in skin VOC profile with exercise.

A stress response scoring system was devised by P. Patel which was based on that of Turner *et. al*., Section 4.4.3, and is reproduced here with his permission. A participant's response to the stressor was scored on the basis of body mass index (BMI) and change in heart rate. The

equation was designed to normalise responses against baseline heart rate measurements so that only change in physiological response influences the score, rather than baseline physical fitness.

$$Stress\ Response\ Score\ (SRS) = \Delta HR\ (\%) \frac{\Delta HR}{HR_{max}}\ BMI$$ Equation 5.1

Table 5.2 Physical stress response scores (SRS) for each participant.

ID	1	2	3	4	5	6	7	8	9	10
SSR	2274	2664	1390	2310	2498	917	2089	2654	3323	3272

To assess the ability of the candidate compound suite to predict the degree of physical stress the proportional response (PR) of the up-regulated compounds at each experimental phase was calculated as shown in equation 5.2. The PR is computed so that an increase in up-regulated compounds and / or a decrease in down regulated compounds will give a larger PR value.

$$PR = \frac{Sum\ of\ up-regulated\ candidates}{Sum\ of\ all\ candidates} = \frac{\sum[\propto 1 \dots 4]}{\sum[\propto 1 \dots 8]}$$ Equation 5.2

Figure 5.7 shows the stress response score (SRS) plotted against the proportional response (PR) for each participant during each of the four experimental phases, A – D.

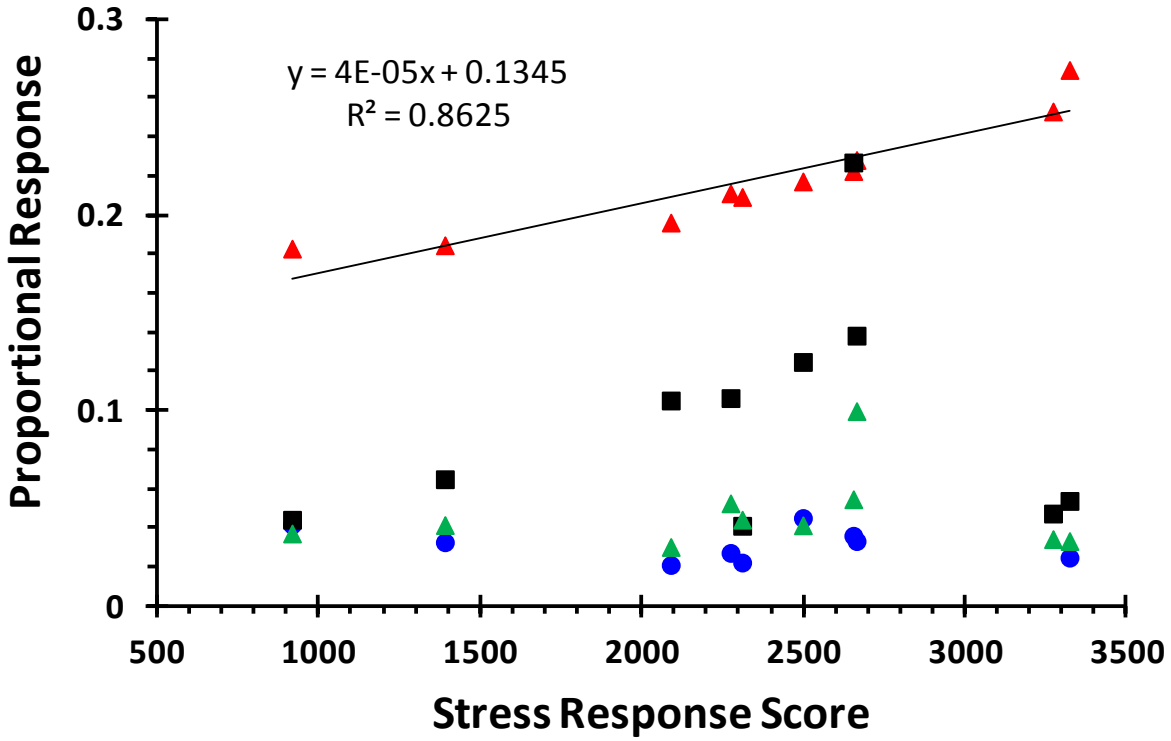


Figure 5.7 Stress response score (SRS) plotted against the proportional response of up-regulated compounds (PR) at each experimental phase. A (blue dots), B (red triangles), C (black squares) and D (green triangles). A trend line plotted for phase B indicates a weak positive correlation between the proportion of up-regulated candidates and stress response score.

Figure 5.7 shows that there is no relationship between the levels of candidate compounds and stress response in phases A or D. Phase B is clearly separated from the other phases while phase C exhibits a mix of behaviors. A weak positive correlation between *PR* and *SRS* is evident in phase B and implies that the *PR* may offer a measure of physical stress.

The stress response score derived in this study is based only on changes in heart rate and so it is, of course, entirely possible that Figure 5.7 simply describes the ability of the candidate compounds to predict an elevated heart rate.

5.3.2. The effect of physiological stress on VOC profiles from human saliva

Saliva VOC samples were obtained following the protocol described in Section 3.3. The saliva sampling protocol requires the participant to hold the sampler in their mouths for the duration of the sampling period, see Section 3.3, the risk to the participant was deemed too high to perform this sampling during the exercise phase, Phase B.

The exercise phase was excluded from the saliva study, reducing the number of observations to 3 per participant: baseline, A, and recovery phases C and D. It is interesting to note here that drooled saliva samples from participants post-exercise took up to 3 times longer (>10 minutes) to collect and for 2 volunteers it was not possible to obtain sufficient drooled saliva for

elements and non-volatile compound analysis in all phases. Whilst the ability to standardise sampling times and collect a full sample with the *in-situ* sampler offers unique advantages over passive drool the level of oral hydration is perhaps a factor that should be considered with this sampler – is there a point at which only the surfaces of the oral cavity are being sampled and if so, to what degree will this alter the physical chemical processes governing sampling?

A malfunction of the filament in the ion source of the mass spectrometer during the analysis of a sample from participant CZ03 necessitated the removal of data from this participant from multivariate analysis. A pareto-scaled data matrix of 144 variables and 18 observations, derived using the workflow described in Section 3.5, populated with peak area responses from the 9 Phase A and 9 Phase C observations was subject to PLS-DA. The resultant S-Plot is shown in Figure 5.8. Seven variables from the extremes of the S-Plot were selected as candidates differentiating between participants in the baseline state, and those recovering from a physically stressed state.

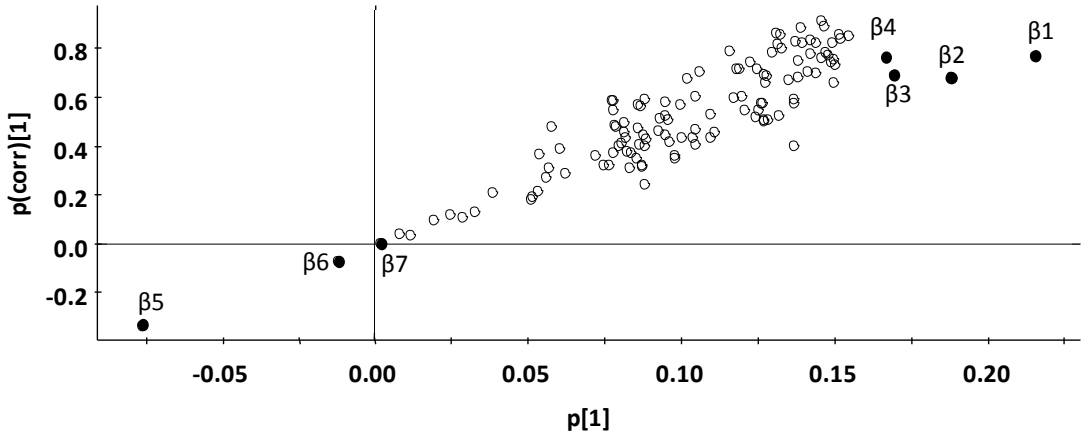


Figure 5.8 The loadings S-plot generated from the PLS-DA model for the metabolomic comparison between pre-exercise (Phase A) and post exercise (Phase C) saliva VOC samples. The variables highlighted (solid dots) at the extremes of the S-plot indicate potential markers sensitive to the exercise intervention.

The candidates were tentatively identified based on spectral matching between their deconvolved mass spectra and the NIST mass spectral database (NIST 11) and a satisfactory match between the experimentally determined and published theoretical Kováts retention index (NIST 11). The criteria for these matches are detailed in Section 3.5.4.

Table 5.3 lists the library entry for each candidate marker, as described in Section 4.3.3, and the tentative identification.

Table 5.3 Tentative identification of exercise sensitive saliva compounds β1-7, including deconvolved mass spectra of the unknowns, forward (F) and reverse (R) NIST spectral match factor and observed (O) and expected (E) retention indices for unknown and library match respectively.

Ref	Name / Library Entry	Deconvolved Mass Spectra	N (F/R)	RI(O/E)
β1	Undecane* Sa-1110-57-43-71-85	57 (999), 43 (900), 71 (500), 85 (293)	840 / 833	1110 / 1100
β2	Butyl Carbitol Acetate Sk-1405-87-57-43-101	87 (999), 57 (999), 43 (950), 101 (200)	890 / 865	1405 / 1408
β3	2, 3 – dimethyl pyrazine Sk-904-67-108-52-66	67 (999), 108 (999), 52 (105), 66 (100)	893 / 888	904 / 911
β4	Methyl-pyrazine Sk-802-94-67-53	94 (999), 67 (500), 53 (150)	955 / 907	802 / 807
β5	Undecanal Sk-1346-57--43-55-82	57 (999), 43 (950), 55 (700), 82 (500)	930 / 932	1346 / 1305
β6	(E) – 2 – Tridecen-1-ol Sk-1550-57-82-55-67	57 (999), 55 (530), 82 (400), 67 (350)	855 / 863	1550 / 1564
β7	3, 4-dimethyl -2-hexanone Sk-836-43-72-57-85	43 (999), 73 (430), 57 (150), 85 (50)	729 / 805	836 / 824

* Assignment confirmed with standard.

Unsupervised PCA was applied to a secondary matrix constructed of peak area responses for the seven variables (candidate compounds) in the three experimental phases (27 observations) the scatter plot generated is shown in Figure 5.9 and shows that these compounds differentiate between the saliva VOC profiles recovered across the experimental phases.

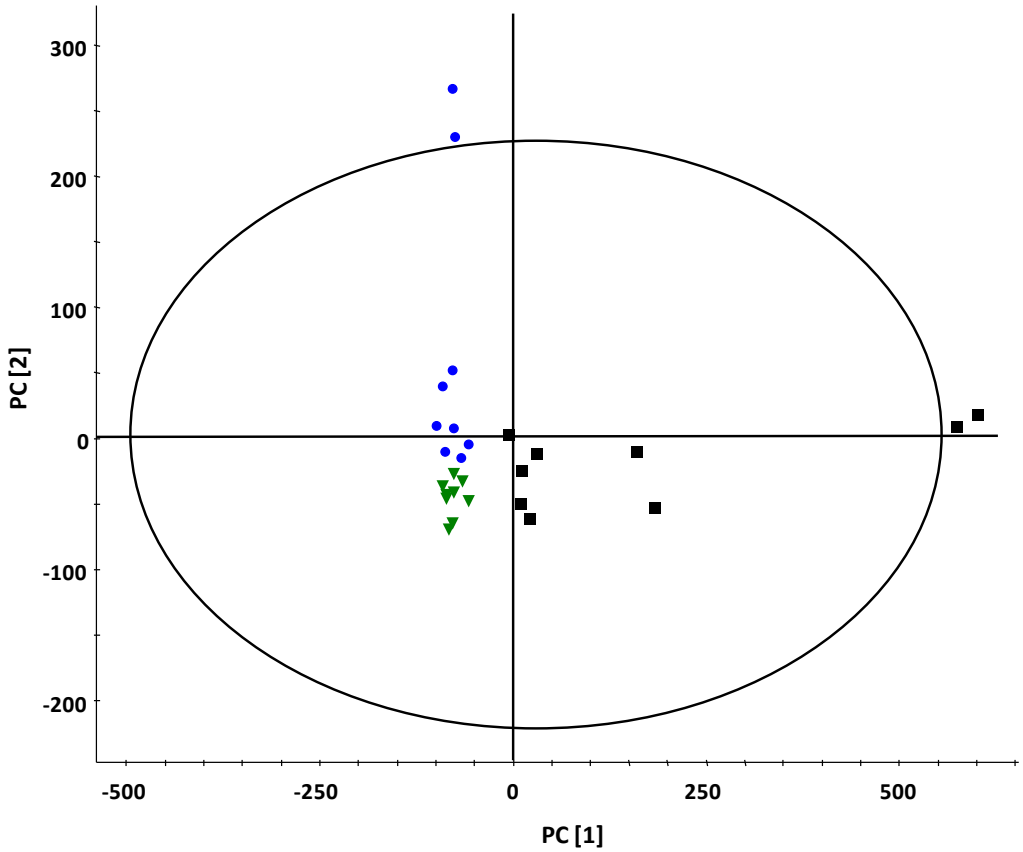


Figure 5.9 Unsupervised principle component analysis (PCA) of seven candidate exercise sensitive VOC selected from PLS-DA of saliva VOC samples. Blue dots show the pre-exercise baseline measurements (Phase A), black squares show the first recovery stage (Phase C) and inverted green triangles show the second recovery phase following on from Phase C (Phase D).

Principle components 1 and 2 identified here exhibit 51% and 20% respectively of the total explained variance of the data set across all 3 experimental phases. The perturbation of the saliva VOC profile with physiological stress is evident in the first recovery phase, C, and in D. The scores plot for skin VOC profiles from phases A and D showed no separation whereas for saliva there is distinct clustering between baseline and second recovery phase.

Two observations lie outside the 95% confidence limits for the PCA model as defined by a Hotelling's T2 ellipse in both the baseline and the first recovery phase, these correspond to the same two participants in both phases; identified as CZ06 and CZ08. Interrogation of each variable reveals these participants to have levels of β5 greater than 3 standard deviations from the mean in baseline measurements and β1 the same in recovery 1; this can be clearly seen in the variable plots shown in Figure 5.10. At this time there does not appear to be a link between these levels and any meta data collected during the study visit. It is interesting to note however, that β1 has been confirmed as undecane whilst β5 has been tentatively identified as undecanal; it is not the focus of this study to determine the biological origin or metabolic

pathways associated with the candidate physical stress sensitive compounds, however it would be interesting to explore any correlations between these compounds in other samples from this study.

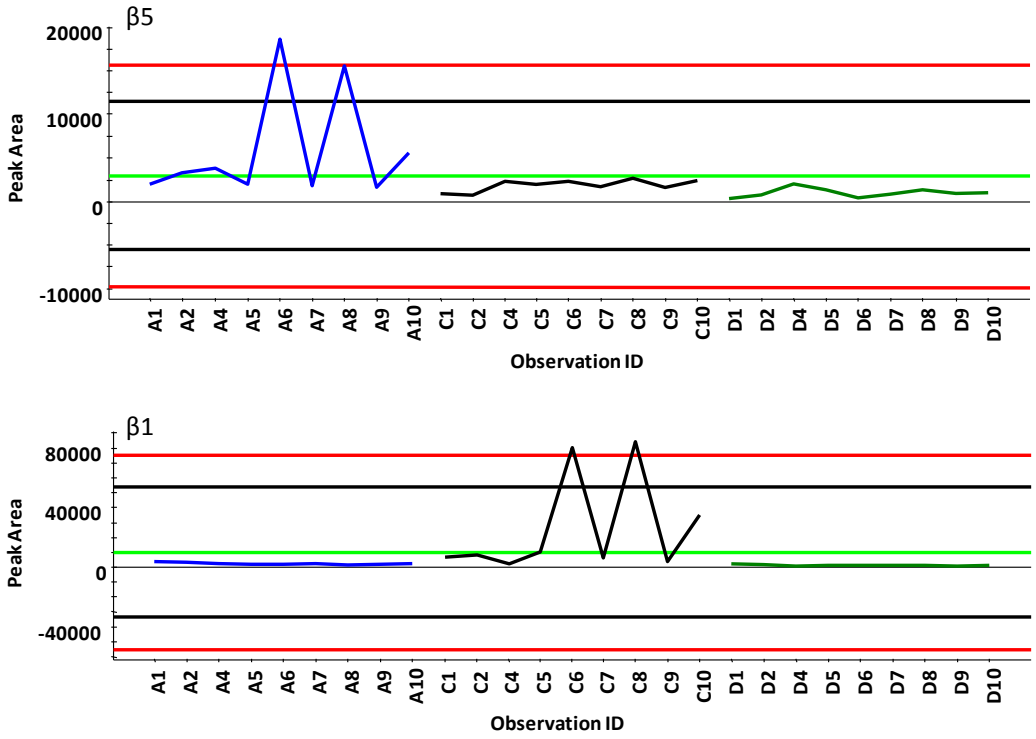


Figure 5.10 Plots of peak area against observation identity (where the letter denotes experimental phase and the number participant ID) for outlier exercise sensitive saliva VOCs β1 and β5 identified in Figure 5.9. The horizontal lines represent limit values with green; the mean peak area, black; 2 standard deviations and the red; 3 standard deviations. Whilst the population shift between the phases can clearly be seen there are two outlying observations for each variable corresponding to participants 6 and 8.

The two trends observed with skin VOC samples: perturbation followed by relaxation to baseline within 60 minutes post exercise and perturbation without relaxation to baseline are also evident for saliva VOC profiles, although without a sample for Phase B it is difficult to directly compare the kinetics.

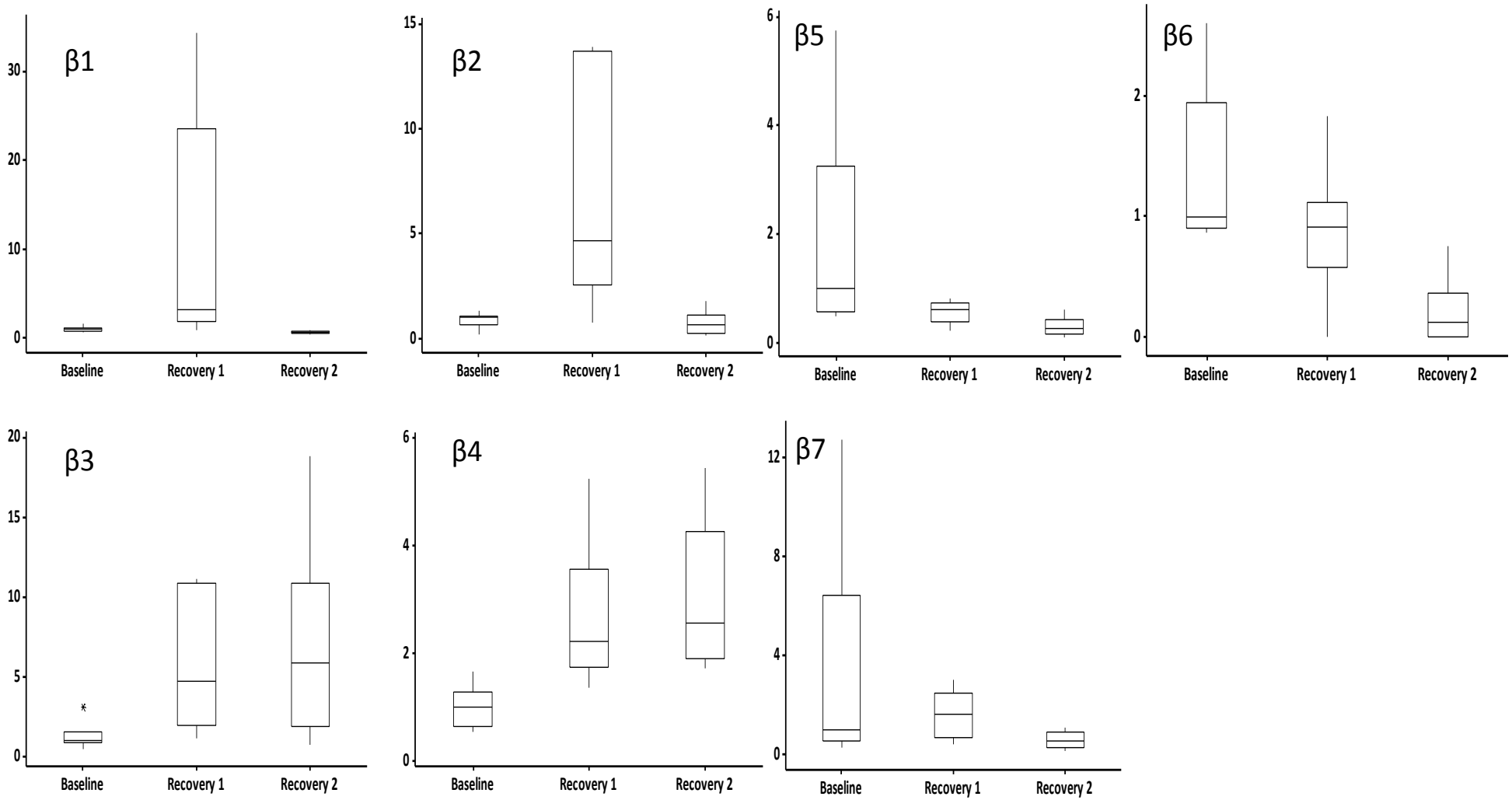


Figure 5.11 Box and whisker plots of peak areas for the 7 exercise sensitive saliva VOCs identified by PLS-DA. Peak areas have been normalised so that the median of the baseline phase is equal to 1. Compounds β1 – β4 are up-regulated in the first recovery phase with respect to the baseline and β5 – β7 are down-regulated.

Up-regulated β1 and β2 increase post – exercise and return to baseline levels in the second recovery phase, β3 and β4 continue to increase across the monitored recovery periods. In contrast to observations in skin profiles none of the down-regulated compounds β5, 6 and 7 shows any indication of returning to the initial baseline levels. Calculated as described in Section 5.3.1 plots of *SRS* against *PR* for phases A, C and D show no correlation between the levels of saliva candidates and the degree of physical stress as described by the stress response score.

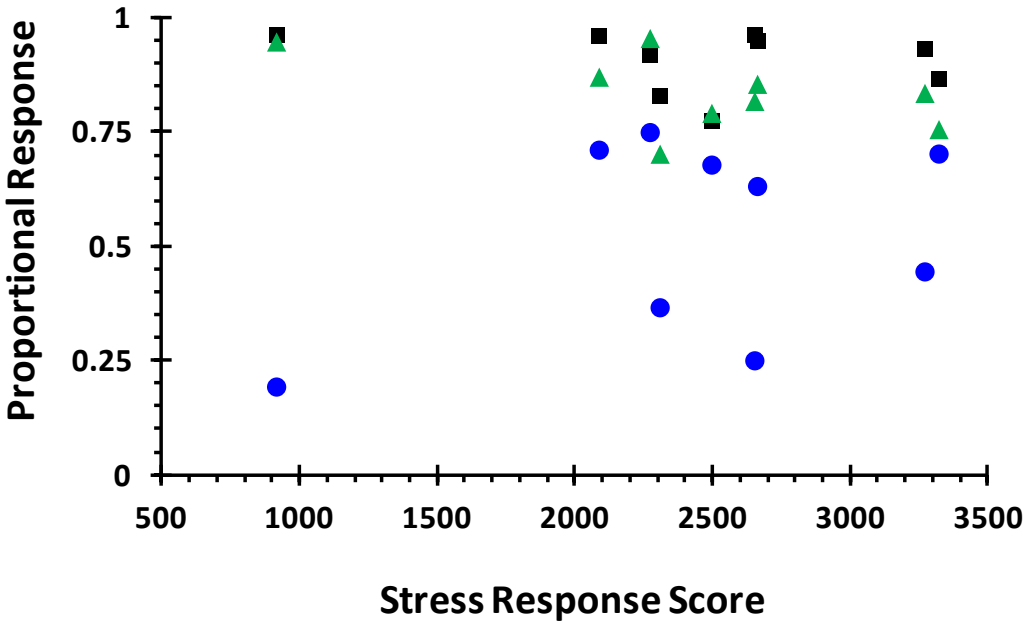


Figure 5.12 Stress response score (SRS) plotted against the proportional response of up-regulated compounds (PR) at each experimental phase. A (blue dots), C (black squares) and D (green triangles).

The lack of correlation between saliva candidates and the stress response score could lead to one of two conclusions; these compounds do not indicate physical stress or the stress response score, being based primarily on ΔHR, in fact only gives an indication of increased heart rate. Without saliva samples from the exercise phase to compare with to explore this any further would be speculation.

5.3.3. Comparison of skin and saliva VOC profiling for tracking metabolic changes associated with physical stress

Skin and saliva VOC profiles appear to change as a result of physiological stress, with two types of change observed. The first change type appeared to be related to the change in heart rate. Compounds tentatively identified as 9-octadecanal and E-2-nonenal in skin and undecane and butyl carbitol acetate in saliva increased, (Figure 5.4 and 5.11) during the exercise phase (Phase B) and then reduced during the recovery phases towards the starting conditions.

Conversely n-decanoic acid and palmitoleic acid in skin reduced during exercise and then increased towards the starting conditions Figure 5.4, no such behaviour was observed for the saliva candidates.

The second type of behaviour was not correlated to heart rate and the changes in candidate marker levels developed beyond the duration of the exercise phase and did not return to the starting conditions during the duration of the test. For instance 2-butyanoyl furan in skin and 2, 3 dimethyl pyrazine and methyl pyrazine in saliva increased , and 2, 3-dichlorobenzoic acid and octanoic acid in skin and undecanal, E-2-tridecen-1-ol and 2, 4 dimethyl hexanone in saliva decreased relative to the starting conditions (Figure 5.5 and 5.11).

Different candidate stress sensitive compounds were identified in skin and saliva VOC profiles, the following sections explore the relative merits of the two approaches and the potential implications of the differences in candidate marker panels.

5.3.3.1. Implementation of sampling and analysis protocols

Both approaches were well tolerated by the participants in this study, neither caused any pain or discomfort and researchers found them straightforward and easy to implement.

Both sampling techniques offer the same advantages in that the sampling methodology is inherently simple, and skilled researchers or clinical staff are not required for sampling. The sample medium is re-usable, samples require no particular treatment before storage and are easy to transport.

While sampling from the skin surface is straightforward it may be difficult to standardise the skin type/condition for every participant. Skin is not necessarily the same in character at the same location in all the individuals in a cohort. The effect of freckles, moles, scars or other skin lesions on the VOC profile collected from the skin has yet to be described. Candidate sites for sampling that appear to be consistent between individuals need to be tested and verified, from the observations in this, and other as yet unpublished studies. The suggested sampling sites are the forehead, abdomen and inner forearm. Whether the most consistent sampling sites also provide the most relevant information also requires further characterisation.

The oral cavity is a more well-defined area in terms of reproducibility in sampling site between participants and subject to less exogenous interference and in these studies participants were excluded if they presented with any oral hygiene problems. Clearly the utility of studying the VOC profiles associated with oral lesions, and periodontic disease as well as poor personal hygiene is a logical development of this study. One that might yield useful diagnostic markers as well as identify the potential confounding VOCs associated with such conditions.

The skin sampling protocol followed in this study requires a sampling duration 6 times as long as the saliva sampling. Although this may ultimately be optimised for more targeted studies following the current methodologies saliva sampling offers greater time resolution than skin sampling. Conversely as the Skin Patch can be comfortably and securely worn, out of sight if necessary, participants are able to be sampled whilst carrying out specific tasks in the study protocol, such as the exercise intervention described here, or simply continuing with their normal daily tasks with minimal interruption which cannot realistically be performed while saliva sampling is in progress.

The nature of the skin sampling allows duplicate or even multiple samples to be obtained simultaneously from one participant, whether this is at a range of body sites or to provide replicate measurements, it is difficult to comfortably obtain duplicate saliva samples simultaneously using the current sampler design.

In use neither sampling approach has a decisive advantage over the other, and, if the VOC profiles are complementary then using both techniques simultaneously with the same participant is not a burden. The non-invasive, injury/trauma-free, embarrassment-free nature of both sampling techniques combined with the low equipment/consumable overheads and straightforward sample storage conditions offer distinct advantages over breath, blood, urine and faeces for large scale, remote investigative or diagnostic studies.

5.3.3.2. Which technique generated the highest quality data?

Visual inspection of skin and saliva VOC profiles reveals information-rich chromatograms, with the skin VOC profile being the more complex with wider range of peak intensities. Figure 5.13 compares the skin and saliva profiles and it is immediately apparent that the Skin Patch yields several orders of magnitude in peak intensity. It is necessary to magnify the saliva profile ten times before a similar level of complexity is discerned, which makes the levels of artefacts from the PDMS samplers of greater concern. The higher recoveries from the Skin Patch may be attributed to: the larger volume of PDMS and therefore increased phase ratio relative to the saliva sampler; the larger surface area in contact with the sample; the nature of the Skin Patch sampling mechanism from the lipid layer on the skin surface providing enhanced pre-concentration; or the wider range of VOCs on the skin and the high concentrations that arise from topically deposited materials; or a combination of some or all of these factors.

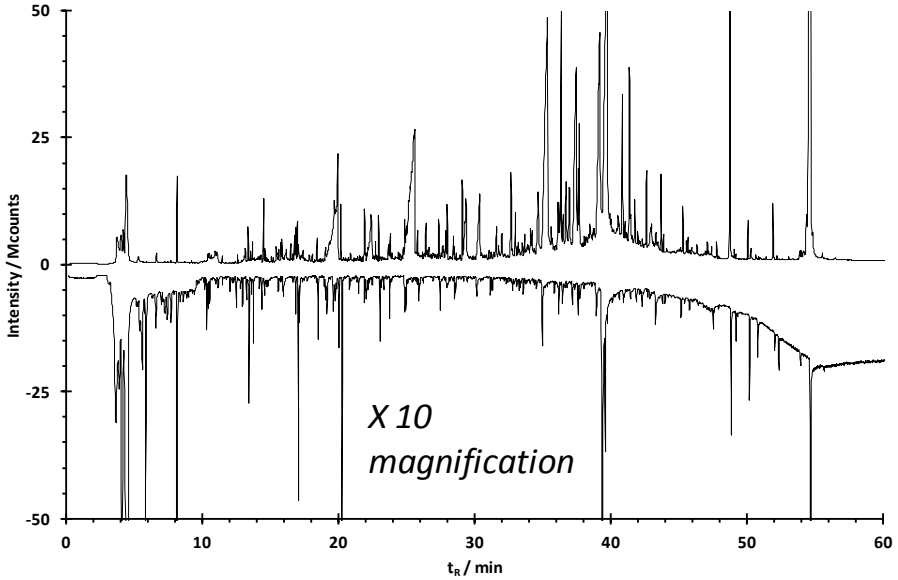


Figure 5.13 **Example skin and saliva VOC TIC chromatograms from a single participant. (Top) Example skin VOC profile and (inverted) example saliva VOC profile from the same participant shown at 10 fold magnification..**

When comparing the two techniques the implications of the higher recovery with the skin sampler should be explored; higher recoveries and concentration ranges have been observed with the Skin Patch – does this mean, therefore, that the skin profile offers a more efficient solution to non-invasive sampling?

It is evident from the skin profile shown in Figure 5.13 that a number of peaks are chromatographically overloaded and as a result may not give an accurate representation of the levels sampled by the Skin Patch, this can be easily overcome by introducing a split into the thermal desorption method. This comes with the sacrifice of the lower intensity, but not necessarily less significant, compounds. Re-collection of the split flow onto a tube packed with an appropriate sorbent is an elegant solution to the problem of samples within which a large concentration range is spanned. The initial sample is run at a 'high' split so as not to overload the high level compounds, the split flow is collected onto a sorbent tube which can then be analysed with a 'low' split for maximum sensitivity.

Recollection of the split portion of a skin sample would allow retention of the maximum amount of useful information from these complex samples. Preliminary observations in developing such a method using Skin Patch samples has shown the high inter-sample variability with respect to the presence and levels of compounds in the profile to present significant difficulties when choosing these 'high' and 'low' split ratios as the optimum tends to be somewhat sample specific. Further, introducing additional desorption steps may introduce selectivity and losses of the more active component and will double the sample analysis time for the study.

While the saliva samples have been shown to deliver lower levels of compounds than the skin, the necessity to run each skin sample twice, once with a high and once with a low split,

significantly increases the work load associated with analysis and data processing for the skin compounds if the full range of VOCs detected was to be available for quantitative analysis, somewhat negating this advantage of the skin sample over the saliva.

It is particularly interesting to note that whilst each skin profile on average yields 3-400 resolved and un-resolved VOCs and each saliva sample typically yields in the order of 200 the resultant matrices from this study include 107 variables from the skin and 144 from the saliva samples. The criteria for inclusion to the matrix in this study was that a compound was required to be present in a minimum of 30% of all observations, the implication of this then is that whilst the skin profiles are comparatively rich in information the inter-sample variability is so great that less compounds pass the criteria for inclusion into the matrix for subsequent consideration as candidate markers than from the saliva. When the relative biological functions of skin and saliva are considered this is not a surprising observation. As a barrier the skin is subject to a range of exogenous influences from the environment, clothing and personal care products – the story of a person's day may very well be written in the VOC profile on their skin. While this provides a highly personalised and interesting set of compounds in this class of sample, potentially highly valuable in forensic – type applications, these exogenous inputs add a level of confounding factors when prospecting for markers of a metabolic state that do not need to be considered to the same extent with a saliva sample. There will still be exogenous inputs and confounding factors in a saliva sample, however saliva is replenished more often and it is generally easier to control the exogenous factors with a participant's saliva by inducing fasting and restricting oral hygiene and personal care products.

5.3.3.3. The best measure of physical stress?

This study was designed to perturb the metabolism of healthy young adults and assesses the relative merits of the skin and saliva VOC sampling approaches in tracking such a change. In this case both approaches have presented candidate compounds that differentiate between baseline measurements and measurements during exercise and the following recovery. When the biological pathways and physical chemical processes involved in creating the endogenous portion of skin and saliva VOC profiles are considered, discussed in detail in Sections 1.3.2 and 1.4.1, it is not surprising that different suites of compounds were found to differentiate between the stress states.

This raises the question; does one provide a better indication of physical stress than the other, and if so, is this likely to extend to the measurement of other metabolic or disease states?

Using the PR value, the relative response of up-regulated compounds described in Section 5.3.1, receiver operator characteristic (ROC) curves were constructed to determine how well the stressed state can be predicted during each experimental phase. Table 5.4 summarises

the area under the curve (AUC) values for each sample at each phase. A perfect ROC curve, i.e. a good prediction of the stressed state, has an AUC of 1, a 'useless' curve AUC ≤0.5 [188].

Table 5.4 Summary of area under the curve (AUC) values for ROC curves predicting stress in each experimental phase with standard error (E_{std}) and upper and lower bound confidence intervals ($Conf_U$ / $Conf_L$)

	AUC (E_{std}, $Conf_U$, $Conf_L$)	
Phase	Skin	Saliva
B	1.00 (0.00, 1.00, 1.00)	N/A
C	0.97 (0.32, 1.00, 0.97)	0.95 (0.53, 0.85, 1.00)
D	0.75 (0.11, 0.97, 0.53)	0.99 (0.2, 0.95, 1.00)

The relative levels of up and down regulated candidate markers for both skin and saliva provide good prediction of physical stress when compared to the pre-exercise baseline state. Skin VOC profiles appear to provide a less accurate prediction of physical stress with each recovery phase, while stress prediction with candidate saliva markers appears to remain accurate up to one hour after exercise had finished. This observation is supported by the accompanying non-volatile and elemental saliva studies which also showed measurable profile differences in saliva samples from Phases A and D [46].

There are several implications that may be considered from this; Skin VOC profiles are more susceptible to short term physiological changes than saliva. Metabolic perturbations affecting saliva VOC profiles take longer to recover than skin. Large changes in compounds correlating with a change in heart rate are dominating the skin VOC profiles and masking contributions from markers that may show the same behaviour as those found in saliva [46].

Both approaches offer a solution enabling VOC profiles from unstressed, stressed and recovering individuals to be distinguished, but it is not clear whether one provides a superior measure of physical stress. The data presented here indicates that saliva profiles may react more slowly to an intervention and that such changes take longer to return to baseline levels; an advantage perhaps for biomarker discovery but also an additional source of confounding factors that will need to be considered going forward with this technique.

5.4. CONCLUSIONS: A DIAGNOSTIC OF PHYSICAL STRESS?

5.4.1. Complementary data sets

Skin and saliva VOC profiling in a combined pilot metabolomic study has revealed measurable changes due to an exercise intervention. Furthermore the advantages of the sampling techniques over those for blood, breath, urine and drooled saliva have been highlighted; the sampling methodologies are inherently simple and skilled researchers or clinical staff are not required for sampling. Furthermore the sample media are re-usable and samples require no particular treatment before storage and are easy to transport.

The overriding trend in the skin response to physiological stress was a maximum perturbation during exercise and a relaxation back to baseline state at the end of a 1 hr recovery period while the saliva response did not relax to baseline in this timeframe. It is clear that different underlying metabolic and physiological responses contribute to the VOC profile for each sample and while different compounds were found to differentiate between adult males who were stressed and those who were not for the two sample types, a candidate diagnostic for physical stress was obtained from them both.

A correlation between global averages for peak area response and change in heart rate for skin VOC profiling, which was not observed in saliva profiles, indicates that physical responses may have a dominant effect on the profiles obtained.

There is no clear indication that one sample type offers distinct advantages over the other in implementation, data quality or diagnostic potential. Simultaneous sampling from a single participant is straightforward and efficient, therefore at this stage the logical conclusion is to consider these sampling methods as complementary in future studies.

Overall this study has shown the Skin Patch and saliva sampler and the workflow for VOC profiling in metabolomic studies to perform well and distinguish between surrogate disease states: stressed and relaxed.

5.4.2. Next steps

The potential future directions in evaluating and exploring skin and saliva VOC profiling for in-field and in-clinic screening techniques are virtually limitless and will be discussed in detail in Chapter 6.

The next steps for this particular intervention involve further collaboration with experts in both exercise physiology and chemometrics. Physical stress, as the average person may understand it, seems to be a subjective concept and a baseline level of fitness will have a marked effect on how ‘stressed’ an individual becomes. In repeating this study on a larger scale consultation with exercise physiologists on selecting a more homogenous cohort in terms of baseline fitness and body mass index and in devising an objective measure of physical stress would reduce the subjectivity and perhaps enable a degree of stratification in the data sets: can the degree of physical stress be predicted using skin and saliva profiling?

Larger, as well as more homogenous, cohorts will enable more powerful statistical analysis and collaboration with chemometricians is the next step in handling such complex data sets on a larger scale. Further investigation of the two types of behaviour observed with stress; relaxation to baseline or not, and the potential correlation between change in heart rate and some skin

VOCs are both important next steps in understanding the relationship between skin and saliva VOC profiles.

Finally, incorporation of skin and saliva samples into a combined data set in the first instance, followed by inclusion of blood, breath and urine VOC profiles as well as elemental and small molecule data will provide invaluable insight into the relationship between the sample types and a step towards linking the metabolic profiles associated with each.

6 CONCLUSIONS AND FUTURE DIRECTIONS

6.1. THESIS REVIEW

Successful development of skin and, or, saliva sampling and the accompanying analysis and data-modeling have the potential to lead to non-invasive *in-vivo* methods for biomarker discovery and clinical screening, hence the underlying thesis of this research:

There is diagnostic information in volatile emanations from the skin, and or saliva, of individuals in differing states of health and well-being. Furthermore this information may be captured and exploited in the measurement of human function towards developing the next generation of non-invasive clinical screening techniques.

This research sought to develop, implement and refine sampling protocols and analytical workflows for the non-invasive measurement of human function.

To this end non-invasive samplers have been developed and characterised for skin and saliva samples and tested and evaluated in pilot metabolomic studies. Three principle research objectives were outlined:

Objective 1: Develop and characterise PDMS-based non-invasive sampling techniques for human skin and saliva.

Objective 2: Critically evaluate and optimise existing workflows for pilot volatile metabolomic studies for the application to skin and saliva sampling.

Objective 3: Evaluate the performance of the samplers and workflows in pilot metabolomic studies with healthy young adults subject to stress interventions.

6.2. CONCLUSIONS

6.2.1. Objective 1: Develop and characterise PDMS-based non-invasive sampling techniques for human skin and saliva

6.2.1.1. Skin sampling

The PDMS Skin Patch has been characterised previously and demonstrated in some small, target led studies [34] [111] [135]. This research has developed robust workflows for sampler preparation and sampling and 2 areas of control have been highlighted as essential to the reliability of this approach:

- PDMS conditioning
- Sample storage.

A poorly conditioned Skin Patch has the potential to introduce hundreds of nanograms into the analytical system, and this would not only confound the analysis of endogenous VOCs but it could contaminate instrumentation. The conditioning protocol that was developed comprised sonication in methanol, drying at 190 ºC under vacuum, 1 > mbar, and conditioning at 185 ºC in 100 ml min^{-1} of clean helium carrier gas. This protocol gave reproducible blank GC-MS profiles that contained a series of PDMS derived siloxanes and were otherwise contamination free; the siloxanes were present in all blanks albeit at different levels between Skin Patches.

As well as careful conditioning and preparation of the Skin Patches the cotton pads used to secure the Skin Patch to the skin surface also needed to be conditioned. Failure to do this would also result in unacceptable levels of exogenous contamination in the subsequent analysis for un-targeted studies, and a step was introduced into the sampler preparation work flow to include vacuum conditioning of the cotton pads at 70 ºC for 4 hr.

Stabilised samples mean that they can be stored long enough to enable efficient and cost effective analytical campaigns to be planned. Previously published work indicated a loss in sample integrity above 24 hours storage at 4 ºC, up to 300 fold increases in response to a sub-set of monitored VOCs were observed and attributed to bacterial metabolism [101]. Lower storage temperatures were tested and it was demonstrated that VOC profiles skin samples to be stable up to 21 days at -80 ºC.

With these changes a Skin Patch PDMS based sampling workflow was described that was suitable for use in pilot untargeted VOC profiling studies of skin.

6.2.1.2. Saliva sampling

The complexity and instability of saliva as a sample medium has been remarked upon extensively in the literature, and many of the previously published work flows, methods and protocols do not address the physical chemical attributes of VOCs [47]. A new *in-situ* VOC sampler for salivary VOCs, sampling directly from the oral cavity, using a polydimethylsiloxane (PDMS) based sampler has been developed and demonstrated with *in-vitro* and *in-vivo* characterisation studies [47]. Further, comparison with passive drool saliva collection revealed that this approach delivered substantial improvement in the recovery and reduced variability of VOCs from saliva. As with the Skin Patch careful conditioning of the PDMS samplers at 185 ºC in 100 ml min^{-1} helium produced blanks in which the only detectable features were siloxane artefacts from the PDMS. An extended storage study was not conducted for saliva samples and will be an essential next step in further development of this methodology.

This approach to *in-situ* saliva VOC sampling appears to have significant analytical utility for the study of volatile signatures in human saliva, and this was demonstrated in a pilot metabolomic

study where a dataset, complementary to accompanying skin samples, provided a differentiator between stressed and un-stressed individuals.

6.2.2. Objective 2: Critically evaluate and optimise existing workflows for pilot metabolomic studies for application to skin and saliva VOC profiling.

Recently a work flow has been proposed to address the variability in exhaled breath analysis and the lack of standardisation and unified methods for such studies [70]. The authors claimed that such work flow based approaches enabled straightforward inter-laboratory and inter-study comparison and data consolidation. Such a development was seen to be beneficial in the acceleration of the application of exhaled breath analysis in discovering and validating VOC markers of disease. The current study has translated this approach to skin and saliva VOC analysis and has expanded the benefits of standardised sample processing, data acquisition processing and modelling to enable the construction of complementary data matrices from different sample types; providing a platform for integration of data from different sample types, studies and laboratories into a single statistical analysis in the future. Indexing of VOCs by study, sample matrix, retention index and deconvolved mass spectrum facilitates the consolidation of data and learning across the studies in this, and future, research to produce the first version of a database for biological VOCs and move this research one step closer to the description of a human volatalome.

Skin and saliva samples are more complicated than breath samples; typically breath compounds span 2 – 3 orders of magnitude while skin profiles in this thesis have exhibited up to 4 orders of magnitude between responses in a single profile. By simply observing the shape of skin and saliva profiles in Figures 4.9 and 5.12 the higher recovery of less volatile compounds from skin and saliva compared to breath is evident. Skin, and to a lesser extent saliva, profiles on visual inspection are dominated by features characteristic of polar species, in particular volatile fatty acids, suggesting that the range of chemical functionality recovered from these profiles is more extensive than breath. The additional complexity of such samples makes development of a unified analysis and data processing method challenging and a number of factors within this workflow were addressed to accommodate this. Optimisation of the workflow was required to enable its application to skin and saliva as well as breath which currently requires a significant degree of manual intervention. This has demonstrated the impact of automated data processing on the quality of data output and the necessity for rigorous control of all stages in a study work flow.

Optimisation of dynamic background compensation to prevent truncation of broad peaks, development of two deconvolution methods to accommodate Gaussian and non-Gaussian

peak shapes, application of PDMS-derived siloxanes as internal retention index markers to avoid the complication of internal standard addition and optimisation of existing breath VOC profiling GC methods to improve the accuracy of retention indexing between instruments were all required to incorporate skin and saliva VOC profiling into a workflow with exhaled breath analysis.

The ability to compare data from different sample types has been demonstrated. The exploitation of this will lead to greater understanding of the volatile metabolome and the potential translation of breath derived markers to corresponding skin or saliva markers. This would facilitate simple, self-administrable and wide spread sampling campaigns which are currently difficult with breath sampling.

6.2.3. Objective 3: Evaluate the performance of samplers and workflows in pilot metabolomic studies with healthy young adults subject to stress interventions

The pilot studies in this thesis were designed to invoke a perturbation in the metabolism of healthy young adults and employ the samplers and workflows developed in Chapter 3 to track the emanating VOCs resulting from such a change. Using induced short term stress as a surrogate for disease states the performance of the samplers and workflows could be evaluated and optimised in an iterative process without wasting precious samples from genuinely un-well participants in early stage sampler development.

The skin and saliva sampling protocols and associated work flows were used to study changes in the respective VOC profiles following psychological-stress and physiological-stress interventions. Both types of VOC profiles were observed to change as a result of the intervention, and further new and previously unreported candidates for VOC markers of stress in humans have been tentatively identified; the validation and confirmation of these markers is beyond the scope of the current study.

Both the Skin Patch and the saliva samplers were well-tolerated by participants and were found to be safe and comfortable non-invasive samplers. Strict control of all aspects from ethical clearance through to multivariate statistics enabled the potential of these samplers to provide complementary information to breath samples to be demonstrated. The potential benefits of further larger follow-up studies have also been argued.

6.3. FUTURE DIRECTIONS

The research presented in this thesis has developed, tested and optimised sampling and analytical protocols for prospecting for volatile biomarkers of human function using non-

invasive methodologies. The testing, evaluation and subsequent enhancement of the workflows is an iterative process and the work in this thesis represents the first step towards a robust workflow for metabolomic studies using skin and saliva sampling, facilitating straightforward comparison between sample types, individual studies and even laboratories. Future collaboration with experts in chemometrics will allow complementary data sets generated using these workflows to be integrated for combined analysis.

The next steps for this work can be split into two categories; further improvements to the analytical workflow and its application to more extended metabolomic studies. These directions will be discussed independently of one another.

6.3.1. On-going workflow development

Recently a study of human body malodour by Unilever PLC employed the Skin Patch and the workflows detailed here to sample the axillae of a panel of female participants selected by genotype [189]. The preparation of sampling materials took place in Loughborough, UK, and these were then shipped to a research centre in Manila, Philippines, where skin VOC samples were taken and malodour assessed. These samples were then shipped back to three different laboratories in Europe for analysis. The results from the sample set sent to Loughborough, and analysed by Helen Martin, demonstrate the application of the protocols developed in this thesis to remote, in-field sampling of human skin in a targeted study of malodour and demonstrates the classification of the malodour phenotypes based on skin VOCs [190].

The workflow presented in this thesis has been adapted from a breath VOC specific protocol to accommodate the additional complexity from skin and saliva. The workflow appears to generate complementary data sets from these sample types and enable straightforward inter-sample comparison that should translate easily to inter-study and inter-laboratory sharing of the biological VOC data. This should facilitate the development of volatile metabolome databases and help accelerate and direct research efforts in the identification and validation of biomarkers and move non-invasive screening towards in-field and in-clinic application.

The workflow presented here is by no means prescriptive or definitive, it will be the work of many future studies to identify other challenges that arise from more widespread application in larger cohorts and current work has highlighted gaps in storage stability, internal standard addition, automated data processing and sampling bias that should be addressed before the next cycle of pilot studies.

6.3.1.1. Storage stability

Long-term sample storage is an on-going concern, in metabolomic studies it may be desirable to collect samples over a period of months, or even years, then analyse them in one batch to

minimise variations in performance of the analytical instrumentation. The minimum storage requirement for a sample should allow for transportation from the field when necessary, sufficient time to analyse all samples in a randomised order and allow for a predictable degree of instrument failure and maintenance. Studies in this thesis have shown VOC samples from human skin to be stable for up to 21 days when stored at -80ºC, whereas to date no such studies of saliva VOC samples have been undertaken.

It is clear that more extended storage studies for both sample types at -80ºC will be required before they can be applied to larger scale studies. In Section 3.1.3 it was demonstrated that storage at -20ºC was insufficient to halt the apparent sample degradation causing an increase in levels of 6-methyl-5-hept-2-one beyond 11 days storage and raised the concern that bacterial activity on the PDMS may not be completely halted by low temperature storage and other approaches to sterilise the sample should be investigated.

Potential solutions for storage of such samples focus around creating an environment that does not support bacterial life; Riazanskaia *et.al* proposed irradiating Skin Patch samples with ultra-violet light prior to storage [111], while this may achieve the desired result it will be important to assess the potential for UV-induced reactions by absorbed VOCs. Re-trapping the VOCs onto an adsorbent material presents itself as an elegant solution; the PDMS sampler which plays host to bacterial life is desorbed with high temperature and an inert gas flow, in the same way as during sample analysis, the flow is redirected so that it passes onto an adsorbent tube instead of a secondary focussing trap or the GC column. Provided that the sorbent(s) selected are able to quantitatively trap and release the range of VOCs under study and that the breakthrough volume of the sorbent is not exceeded complete transfer of VOCs from the sampler to the tube can be achieved at the same time as sterilisation with high temperatures and oxygen starvation. Multiple bed adsorbent tubes can be stored at 4ºC for a number of weeks [62] [70] and as indicated in as yet unpublished studies on the storage of breath VOC samples; at -80ºC for up to one year.

This approach offers the advantage that no additional instrumentation is required; however it will be necessary to purchase additional sorbent tubes and conduct a storage study with them.

6.3.1.2. Internal standard addition

The addition of an internal standard to liquid samples has become an accepted stage in automated GC procedures [125] and can be used in targeted studies for quantitation, or in non-targeted studies for normalisation in which the peak area for each compound in the chromatogram is divided by the internal standard peak allowing for correction in instrument performance between samples.

The workflow described in this thesis aims to provide a framework that will allow straightforward integration of data sets from different samples, instruments and laboratories. The addition of an internal standard to allow normalisation across data sets, as opposed to external standards currently employed, would be a highly desirable step.

The challenges associated with reproducibly adding a standard to solid adsorbents has been addressed for adsorbent tube samples by directing a flow of a gas standard onto the sampling end of an adsorbent tube. The gas standard could be commercially available or produced from a permeation source as described by Huxham *et.al* [191]. This can be incorporated into the workflow either pre- or post- sampling, or even both with different standards, providing a standard to validate sampling, storage and analysis.

Adding internal standards to absorbent materials such as PDMS is not a trivial task. Absorption is an equilibrium process so the addition of gas phase standards after sampling is unlikely to yield 100% and reproducible capture of standard compounds in a vapour stream and some desorption of absorbed analytes should also be expected.

Soini *et. al.* embedded standards into the PDMS phase of their 'rolling stir bar' skin sampler prior to sampling [110] by stirring the samplers in an aqueous solution of the standards. The stir bars were then rolled across human skin *in-vivo* to sample VOCs from the skin surface. The authors do not discuss recovery of the internal standards but have shown moderate reproducibility with 14.7 %RSD for C^{13}-benzyl alcohol and 14.3% for 7-tridecanone. The authors state that by embedding the standards inside the polymer sorption material human skin exposure to the standards is minimised, yet no evidence is presented to support this. Internal standards should be chosen to closely match the physical and chemical properties of the compounds under study whilst not being present in samples or ambient air. By this definition the concentration of standard at the skin surface must be zero at the start of sampling, as protocols in this thesis sample skin and saliva for 30 and 5 min, respectively, the sample medium is likely to act as an absorbent to extract the standards from the sampler. The potential for losses of internal standard during sampling renders this approach not viable both ethically and as a means of normalisation between samples.

For untargeted global VOC profiling internal standard addition will offer the benefit of facilitating normalisation, but not quantitation. This raises the question – at what stage of the workflow is it necessary to add an internal standard?

To normalise for instrument performance it is simply necessary to add the standard prior to analysis, a simple method for achieving this would be to add a bed of adsorbent material to the sample tube containing the Skin Patch. A liquid phase standard injected onto the sorbent would be desorbed along with the sample. Adding the standard(s) to the sorbent bed before

storing the sample in the tube would provide an internal standard to asses storage, however losses from the sorbent bed may not reflect losses from the PDMS and diffusion between the two phases must be well characterised.

A second PDMS coupon impregnated with standards added to the thermal desorption tube with the sample would provide an internal standard for the storage and transport process. If this was used alongside the sorbent bed approach different internal standards could then be used to validate sample storage and transport as well as instrument performance moving towards robust, intrinsically valid sampling and analysis protocols for metabolomic studies.

6.3.1.3. Sampling bias

The advantages of using PDMS for non-invasive *in-vivo* sampling were described in detail in Section 2.2 and while it has been demonstrated to perform well for in-vivo sampling in this thesis the hydrophobic nature of PDMS affords a bias against very polar species such a salts and sugars found in skin and saliva. Moderately polar species, fatty acids and alcohols, are often reported in such samples. This is due to their proportional partitioning into the PDMS giving a quantitative, representative profile. It is possible to predict the maximum proportional uptake of a compound into PDMS using its $K_{o/w}$ values from equation 6.1 [134]

Equation 6. 1

$$\frac{m_{PDMS}}{m_s} = \frac{\left(\frac{K_{O/W}}{\beta}\right)}{1 + \left(\frac{K_{O/W}}{\beta}\right)}$$

If we assume a sample volume of 1 ml and Skin Patch with a PDMS volume of 34 µl the equilibrium percentage recovery into the Skin Patch can be calculated. Examples for some compounds reported in skin / saliva samples are included in Table 6.1.

Table 6.1 Example maximum recovery values for PDMS sampling of biological VOCs with differing log $K_{o/w}$ values.

VOC	*log* $K_{o/w}$	Max Recovery (%)
Octane	5.15	99.98
Hexanal	1.78	67.20
1-Nonanol	4.02	99.72
Propanoic Acid	0.33	6.78

The implications of this are not that PDMS cannot be used for polar species, quantitative results have been demonstrated, and only that equivalent sensitivity will not be achieved for polar and non-polar species using PDMS alone [196].

Further work on untargeted global profiling of VOCs from skin and saliva would benefit from a more in-depth evaluation of the sampling phase to enhance the coverage and reduce bias in the sampling step. Polyacrylate and ethylene glycol phases have been compared to PDMS for the extraction of whisky and fruit juices and both showed higher affinities for polar species such as phenols and acids while the ethylene glycol phase, which has a dimethylsiloxane base, gave better extraction efficiency for non-polar compounds than the polyacrylate phase. The authors found that by using the ethylene glycol and the PDMS phase sequentially they were able to achieve an overall analyte profile of polar and non-polar analytes [192]. Silica monolith technology, which allows hybrid sorbents to be formed incorporating octadecyl silica, graphitised carbon or PDMS, has been recently applied to solid phase extraction applications. The high porosity of the monolith provides a naturally high surface area and adsorption and absorption processes can be utilised in one sampling device, extending the range of compounds that are extracted efficiently [193] [194].

While these technologies have been applied to liquid samples so far, the potential to develop a combined sampler employing more than one extraction phase is an exciting and important next step for non-invasive global untargeted VOC profiling by direct contact methods.

6.3.1.4. Analytical instrumentation

The difficulty in finding a single GC column that provides efficient separation and acceptable peak shapes for the wide range of volatility and polarity found in skin and saliva VOC profiles presents a significant challenge in untargeted global profiling. Careful application of deconvolution algorithms with mass spectral data compensates for a significant degree of co-elution. The range of peak shapes and widths from skin VOC were shown in Section 3.5 to present significant challenges for such automated data processing.

One solution for the wide range of functionalities present in these sample types would be to move from a single column to a two-dimensional gas chromatography system (GCxGC) in which two capillary columns with different stationary phases are coupled and the sample directed onto each by backflushing, heart-cutting or thermal or flow modulation. For skin and saliva VOCs the use of a mid to non-polar stationary phase, such as a DB-1 or the DB-5 used here, with a polar phase such as a DB-Wax column may provide improved resolving power and Gaussian peak shapes for all compounds. Automated data processing with GCxGC data may require specialist software and the costs, both for initial instrument purchase and overheads such as liquid cryogen for thermal modulation, may make this approach more applicable for biomarker discovery leading to a simpler targeted approach on a single column system for screening applications.

6.3.2. We don't need your blood: future direction for non-invasive biomarkers prospecting using skin and saliva VOC profiling

The application of the samplers and workflows to metabolomic studies is the logical next step to this research. This will not only improve the understanding of these profile types and the links between them but provide further insight into the development of the techniques themselves. The scope for such studies is virtually unlimited and this section prioritises two of the many potential study types as the next phase for this research.

The first set of studies focuses on characterising and understanding the volatile metabolome. Selection of sampling site for skin VOC studies generally seems to be based on other study constraints and a better understanding of the implications of sample site on the skin profile is essential before this kind of sample can be usefully employed in studies of systemic conditions. Simultaneous sampling of a range of body sites is easy to achieve with the Skin Patch and a 'body mapping' study, perhaps incorporating saliva and breath samples, will be a valuable and highly informative next stage in this research.

Further to this a study, tracking the fate of an isotopically labelled substrate, such as a topically applied ointment or the C_{13} labelled breakfast cereal [195] used by Jonderko *et. al*, across skin, saliva, breath, urine, blood and faecal samples over a defined time period would give an insight into the relationships between the profiles associated with each sample type and be a first step towards finding metabolic links between the profiles.

The second set of studies moves the biomarker discovery aspect of this research onto more real world clinical studies. While ultimately a diagnostic for systemic diseases using such non-invasive samplers would be desirable, the logical first step is to examine the profiles associated with conditions of the skin and oral cavity. Studies of skin cancers, lesions, grafts, ulcers, burns, scars and infections would all benefit from a pain free monitoring technique, while oral cancer, lesions and periodontal diseases are a logical development of salivary profiling. Such studies may also yield useful information on VOCs associated with such conditions that may confound biomarker discovery for other systemic diseases.

6.4. SUMMARY

Mamas *et. al.* present three fundamental questions to critically appraise the contribution of biomarkers identified in metabolomic studies towards clinically useful diagnosis [196];

Can the clinician measure it?
Does it add new information?
Does it help the clinician to manage patients?

If a biomarker does not, or will not, reach these benchmarks then does it really add any value? This philosophy can be extended to non-invasive sampler development; while it is relatively easy to bring a cohort of participants to a laboratory and use a bespoke sampler or a real-time analysis technique to be 'non-invasive', how relevant will this information be in developing a real world clinical diagnostic technique?

The current status of this research is a long way from dip-stick type technology that can be employed in a doctor's office or a remote location to provide an instant diagnostic. The samplers described here are non-invasive, pain-free, simple, self-administrable, portable and clean representing a significant step towards fieldable non-invasive samplers for real world applications.

'We don't need your blood': The outcome of this research is the description of new methods and workflows, the result of continuous evaluation and modification throughout the studies that represent a significant step towards more robust methods for non-invasive marker discovery and diagnostic techniques.

APPENDIX 1: Example application for ethical approval

Appendix 1.a: Generic protocol application

This document and the accompanying supporting documents show examples of those submitted to the ethical advisory committee to apply for generic approval for the sampling technique, study specific checklists were also submitted where sampling required an intervention.

GENERIC PROTOCOL APPROVAL FORM FOR

HUMAN BIOLOGICAL/PSYCHOLOGICAL/SOCIOLOGICAL* INVESTIGATIONS

* delete as appropriate

This application form should be completed for **generic protocols** NOT **individual research proposals**. It applies to procedures/techniques that will be performed on a regular basis and form all or part of subsequent research projects. This form should not be used for one-off research projects.

This application form should be completed after reading the University Code of Practice on Investigations on Human Participants (found at http://www.lboro.ac.uk/admin/committees/ethical/ind-cophp.htm).

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1. Title of Procedure/ Technique

Chemical Profiling of Human Skin.

2. Brief lay summary of the procedure/ technique for the benefit of non-expert members of the Committee

This project seeks to develop advanced sampling and measurement methodologies for measuring human chemical profiles in-vivo. In order to develop sensitive and specific assays to recover key bio markers from the skin surface a novel non-invasive device called "Skin Patch" was developed. The silicon Skin Patch is a thin inert membrane that is placed on the skin surface in order to adsorb all volatile and non-volatile material from the skin.

The Skin Patch was awarded a favourable ethical opinion on the 26th September 2006 by Oldham Local Research Ethics Committee, Reference number:

06/Q1405/34 (Annex 1). The Committee designated this study as exempt from site-specific assessment and this document is designed to update local ethics committees for continuation of this work.

3. Details of responsible investigator (supervisor in case of student projects)

Title: *Prof* Forename: *Paul* Surname: *Thomas*

Department: *Chemistry*

Email Address: C.L.P.Thomas@lboro.ac.uk

Personal experience of and/or training in proposed procedure/technique,

Prof Thomas and his research group designed and tested the sampling patch for use on the skin surface at Manchester Uni. It has proven very successful in sampling volatile organic compounds from skin and is currently being used in prospecting studies for skin cancer, profiling of chronic wounds and human odour profiles.

4. Names, experience, department and email addresses of additional investigators (if known)

Please notify the Committee Secretary when additional investigators are trained in the procedure/technique.

- *Helen Martin, PhD student/researcher, H.J.Martin@lboro.ac.uk*
  Trained at Loughborough awaiting ethics approval to practice, Chemistry
- *Matthew Turner, PhD student/researcher, M.A.Turner@lboro.ac.uk*
  Trained at Loughborough awaiting ethics approval to practice, Chemistry
- *Aditya Malkar, PhD student/researcher, A.Malkar@lboro.ac.uk*
  Trained at Loughborough awaiting ethics approval to practice, Chemistry
- *Pareen Patel, PhD student/researcher, P.Patel@lboro.ac.uk*
  Trained at Loughborough awaiting ethics approval to practice, Chemistry
- *Corrine Burns, Post Doctoral Research Assistant, C.Burns@lboro.ac.uk*
  Trained at Loughborough awaiting ethics approval to practice, Chemistry

5. Location(s) if known

Currently Analytical Research F2.07/F2.08 Chemistry

6. Will any of the participants be from one of the following vulnerable groups?

Children under 18 years of age	Yes ☐ No ☒
People over 65 of age	Yes ☐ No ☒
People with mental illness	Yes ☐ No ☒
Prisoners/other detained persons	Yes ☐ No ☒

Pregnant women	Yes ☐ No☒
Other vulnerable groups	Yes ☐ No☒

7. Will the procedure/technique involve any of the following?

Testing new equipment/drugs	Yes ☐ No☒
Administering drugs (including caffeine)	Yes ☐ No☒
Use of radiation (eg x-rays)	Yes ☐ No☒
Assisting/altering the process of conception in any way	Yes ☐ No☒
Methods of contraception	Yes ☐ No☒
Genetic engineering	Yes ☐ No☒
Will be conducted outside of the UK	Yes ☐ No☒

If yes, please submit a full research proposal to the Ethical Advisory Committee. Generic Protocols cannot be used to cover these activities.

8. Description/outline of procedure/ technique including reasons for performing the procedure/technique

Please refer to Appendix 1b

9. Do any investigators stand to gain from a particular conclusion of the procedure/technique?

No

10. Methodogy (detailed description)

Please refer to Appendix 1b

11. Detail of measurements to be taken

Please refer to Appendix 1b

12. Please indicate whether the proposed procedure/technique:

Involves taking bodily samples	Yes ☐ No☒
Involves procedures which are physically invasive	Yes ☐ No☒
Is designed to be challenging (physically or psychologically in any way)	Yes ☐ No☒
Involves dietary manipulation or supplementation	Yes ☐ No☒
Prescribes intake of compounds additional to daily diet	Yes ☐ No☒

Involves procedures which may cause embarrassment to participants	Yes ☐ No☒
Involves collection of personal and/or potentially sensitive data	Yes ☐ No☒

If Yes - please give details of the arrangements to deal with adverse effects.

No adverse effects foreseen

13. Participant information

Details of participants (gender, age, special interests etc)

Participants are likely to be recruited from cohorts of people who are phenotypically matched and have well characterised disease, metabolitic or environmental factors associated with them for which metabolitic markers on their skin are sought.

Preliminary studies will focus on establishing the boundaries of normal homeostaisis correlated to indicators of vitality. These will recruit from healthy young (18 yr to 35 yr) adults of both sexes.

Future studies may require additonal approval as the research methods are adopted and adapted to serve the needs of other studies within the Loughborough University Research Network.

Please outline inclusion/exclusion criteria to be used in selecting participants for this procedure/ technique.

Inclusion and exclusion criteria are specific to individual studies.

Such studies will be the subject of further ethical approval if indicated by Loughborough University Ethical Clearance Checklist.

Where appropriate please indicate where participants will be recruited from.

Loughborough University Staff and students and their associated social networks.

14. Procedures for chaperoning and supervision of participants during the procedure / technique

1. Briefing and consent.

Volunteers are briefed in private in an annex to F 2.07 with windows in the doors. The researcher explains the process, and answers questions about the procedure during the briefing. Informed consent is taken and an appointment is made for the samples to be taken.

2. Participants questionnaire

The questionnaire is completed in private in an annex to F2.07 with windows in the doors.

3. Skin sampling

This takes place in a screened and designated area in our laboratory F 2.07. Normally one researcher will work with the participant while co-workers will be aware of and observant of the procedure. (They will not necessarily be involved in the sampling procedure but will be briefed and ready to assist if required.) There are always more than two researchers present in the laboratory and lone working is forbidden.

4. Escorting and supervision.

Participants are accompanied at all times while they are in our laboratory.

Participants will be offered a same sex chaperone.

15. Possible risks, discomforts and/or distress to participants

1. Possible Risks

The procedure takes place in a sampling station which is screened and designated area within F2.07. Within this facility the normal hazards associated with our laboratory operations (Mainly accidental splashes and high pressure gas installations) are mitigated. Walking to the sampling station exposes the participant to the normal risks associated with our laboratory operations and personal protective equipment (Safety glasses and laboratory coat) will be issued to them.

It may be possible for a participant to be allergic to the silicone sampling device. Allergic reactions to silicone are rare and, as it is used in cookware, baby bottles, numerous medical applications and even personal care products, it is highly unlikely that a participant would be unaware of this allergy. Participants are informed of the risks and asked to fill in a health questionnaire in which they are asked to detail any allergies, with these precautions the risk of allergic reaction is minimal.(Reference - Medical Device and Diagnostic Magazine. Nov 1999. Charles Heide. 'Silicone Rubber for Medical Applications'.)

2. Discomfort

The sampling patch itself is a small, malleable membrane that we can foresee no discomfort associated with.

3. Distress

A stranger working very close to you may be unsettling.

During sampling, care is exercised to ensure that the participant is comfortable and aware of what is happening. Every action is described and explained before it is undertaken and during the procedure. For example:

"Is it OK for you me place the patch on your skin and cover it with this dressing?"

"Is that comfortable, are you OK to continue with the experiment?"

16. Details of any payments to be made to the participants

No payments are made to participants

17. Will written consent be obtained from participants for the procedure/technique?

Yes ☒ No☐

If Yes, please attach a SAMPLE (generic wording to cover this procedure/technique to be included in main consent form) of the consent form to be used.

If No, please justify.

Please see Appendix 1.d.

18. How will participants be informed of their right to withdraw from, or stop, the procedure?

This is explained during the briefing and in the participant's information sheet (Appednix 1.c).

Before each stage of the procedure the researcher seeks permission from the participant to proceed, typically as part of the explanation and discourse provided about the procedure. For example:

"Could I remove the patch and dressing from your skin?", "Was that OK, would you be OK to give another sample in the same way?"

Finally, as the participant is in control of the device, they are able to remove the device quickly and easily at any time during the process. A reminder of their right to withdraw whenever they wish with no ill consequences is given to the participant immediately before sampling

19. Will the procedure / technique include the use of any of the following?

Audio/video recording	Yes ☐ No☒
Observation of participants	Yes ☐ No☒

If yes to either, please provide detail of how the recording will be stored, when the recordings will be destroyed and how confidentiality of data will be ensured.

20. What steps will be taken to safeguard anonymity of participant . confidentiality of personal data?

All data are anonomised. Participant questionnaires do not contain personal details. A record of the participants attendance is maintained separately and held securely.

21. Will collection and storage of data comply with the data protection act?

Yes. All personal information and details will only be entered onto written consent forms which will be kept in a locked drawer in the chemistry department. All experimental data, questionnaires and outputs will refer to participants only by identification numbers. Only information relevant to the specific study will be recorded and data will only be used in conjunction with the specific study.

22. Insurance cover

It is the responsibility of investigators to ensure that there is appropriate insurance cover for the procedure/technique.

The University maintains in force a Public Liability Policy, which indemnifies it against its legal liability for accidental injury to persons (other than its employees) and for accidental damage to the property of others. Any unavoidable injury or damage therefore falls outside the scope of the policy.

Will the procedure / technique result in unavoidable injury or damage to participants or property?

Yes ☐ No☒

If yes, please detail the alternative insurance cover arrangements and attach supporting documentation.

The university insurance related to claims arising out of all normal activities or the university, but insurers require to be notified of anything of an unusual natures.

Is the procedure / technique classified as normal activity?

Yes ☒ No☐

If NO, please check with the university insurers that the policy will cover the activity. If the activity falls outside the scope of the policy, please detail alternative insurance cover arrangements and attach supporting documentation.

23. Declaration

I have read the University's Code of Practice on Investigations on Human Participants and completed this application.

I agree to provide the Ethical Advisory Committee with appropriate feedback should I need to make any changes to this protocol in light of experience.

Signature of applicant: .....

Signature of Head of Department: .....

Date .....

For all applications:

Please ensure that you have attached sample copies of the following documents to your submission

- Participant Information Sheet
- Informed Consent Form

In addition, please attach sample copies of the following documents if applicable.

- Willingness to Participate Forms
- Health Screen Questionnaire
- Questionnaires and Example Interview Questions
- Advertisement/Recruitment material
- Evidence of consent from other Committees

Appendix 1.b: Sampling protocol

Detection of Volatile Organic Compounds on human skin

Helen Martin, Research Student, Loughborough University

Professor Paul Thomas, Professor of Analytical Science Loughborough University

Professor Colin Creaser, Professor Analytical Chemistry, Loughborough University

Background

It is ten years since the publication of Phillips' landmark review of volatile organic compounds (VOC) as markers of disease[1], since then the continuous development of instrumentation, sampling and information processing techniques has revealed the existence of a dynamic individualised and highly complicated VOC profile associated with every aspect of human life. Our research group has developed expertise in extracting this VOC profile from exhaled breath and from the skin surface and much of our work focuses on creating next generation devices for non-invasive *in-vivo* VOC sampling. This study aims to take one such device that has proven successful on skin[2,3] in other locations and extend it to further studies at Loughborough University.

The current study seeks to discover if relationships between VOC profiles and well-being exist; where well-being may be represented by a variety of non-specific indicators such as induced stress, cardio vascular function, body mass index and respiratory function for instance.

This study will use modern, state-of-the-art chemical detection systems to pinpoint potential biomarkers within the VOC profile and identify them. Identification of such chemical signatures may ultimately provide an objective method for monitoring human vitality.

Hypothesis and aims.

Our research hypothesis is that there is a detectable difference between the chemical compounds present on the skin of participants who enjoy different levels of health and well-being and who engage in different lifestyles. Such chemical differences could be identified as potential biomarkers for varying degrees of vitality.

This generic protocol will collect samples of VOC from the skin surface and chemically analyse them. Using advanced statistical approaches the intent is to establish if it is possible to distinguish between the skin of participants with differing states of well-being. Further the intention is to determine the chemical identity of any candidate markers that are discovered in such studies..

Methods

Setting

A laboratory F 2.07 in the Department of Chemistry equipped with a comfortable seat for the volunteer participants and a table for skin sampling equipments.

Volunteer participants

This generic ethical approval will enable volunteers to be recruited from individuals who are clearly differentiated in their lifestyle or levels of health from the general population. Specific studies will be subject to ethical approval based on individual research proposals.

Prospective participants will be provided with written information about this study and where they are able and willing, informed consent to participate in the study will be obtained. Participants will normally have at least 24 hours to decide whether to participate in the study. Any information relating to personal information about participants will be made available to the researchers by sample codes only, no names or addresses will be used.

Inclusion criteria

- These will be study specific and subject to ethical approval obtained for individual project proposals.
- Age normally ≥ 18yrs. Should participants from vulnerable groups be sought additional safeguards will be implemented that will be described in individual project proposals.

Exclusion criteria

- These will be study specific and subject to ethical approval for individual project proposals.

Sample collection

VOC sampling from skin will employ an inert silicone patch placed on the skin surface, normally on the forearm, forehead or upper back. The sampling patch will be held under a non-adhesive dressing which in turn is held in place by hypoallergenic micro porous tape. The patch will remain on the skin for up to 30min at any one time and multiple patches may be applied in parallel. Once sampling is complete the researcher will remove the patch from the participant and transfer the silicone patch to an empty thermal desorption tube using tweezers. The ends of the tube will be sealed with swagelock caps and samples will be stored at 4°C until analysis.

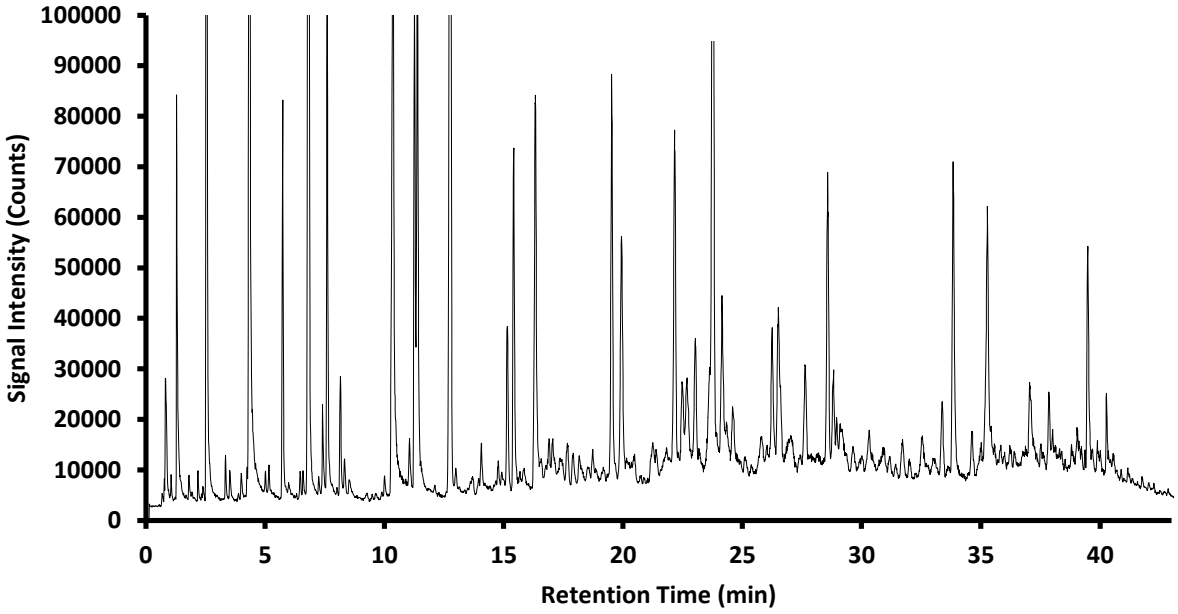
Factors that may affect results may be controlled by asking participants to fast or refrain from smoking for a specified period of time prior to sampling. Any medications that may affect the results will be specified in the exclusion criteria.

Sample processing

The samples will be analysed using a combination of thermal desorption, chromatographic, mass spectrometric and ion mobility techniques. The basic operation of this system is to allow

us to detect all the different chemicals in a breath sample. The ion mobility systems generate three-dimensional surfaces that can be used like a fingerprint for a specific phenotypic state.

The mass spectrometer allows us to identify single components and look for differences in peoples' saliva VOC profiles. It is an extremely powerful tool that will aid us in identifying potential biomarkers. The data it generates is displayed in two forms; a chromatogram and a mass spectrum. An example chromatogram is displayed below in Figure 1.



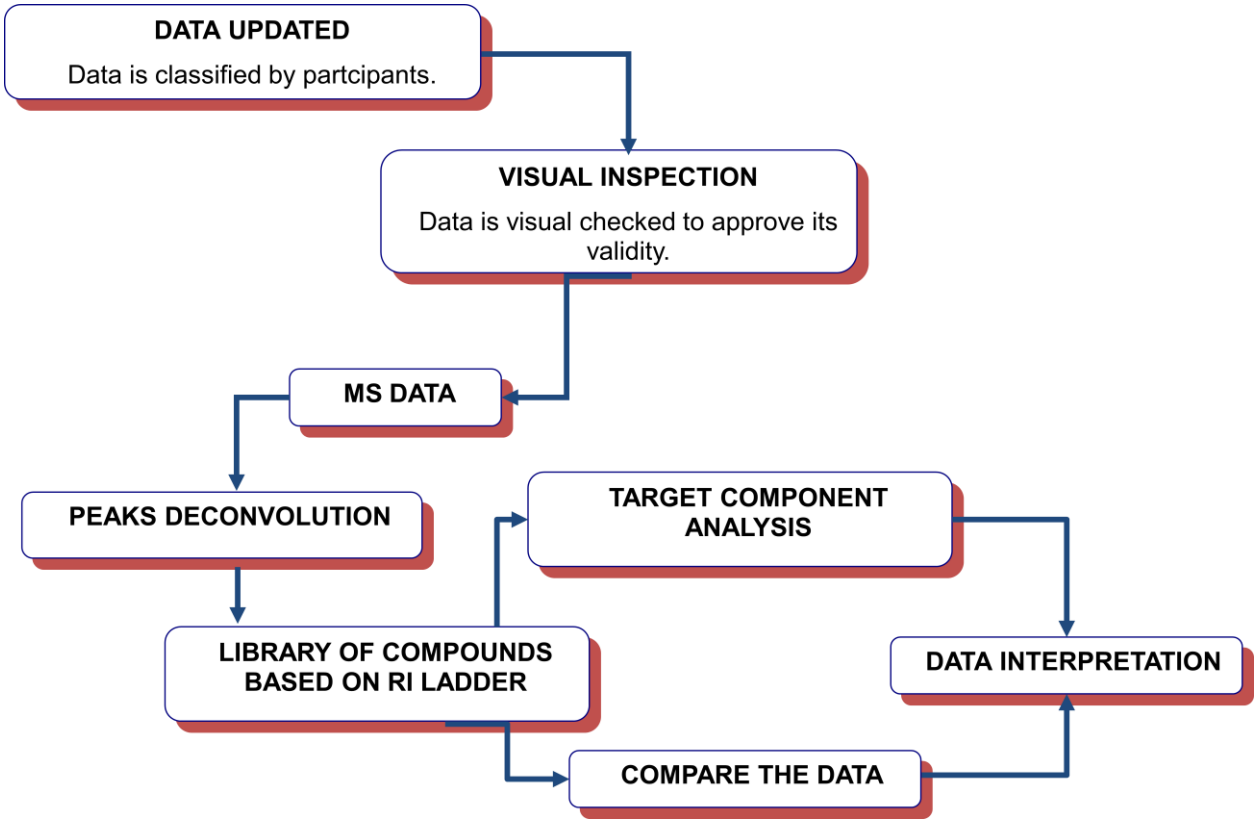
The chromatogram above, in Figure 1, allows the relative concentrations of the different components of the VOC profile to be estimated. Each point on the chromatogram is built up from integrated mass spectra, which may be used to identify the compound.

A range of standard compounds built up into a ladder across the chromatogram can be used to generate a library. Components in the sample VOC profiles can then be referenced against the standards to give a value known as the retention index; this provides another reference point for identification of markers and comparison between samples.

Data analysis

All data will be treated as confidential and rendered anonymous at the point of sampling.

The data flow that we shall use for the analysis of the VOC profiles is detailed in Figure 2.



- Visual inspection of the data will be performed to ensure that no loss in sensitivity has occurred and no contaminant artefacts are present.
- Deconvolution of mass spectral data will be performed using state-of-the-art software to generate VOC peak lists for each profile.
- Components will be incorporated into a library set up for the study based on their retention index and where possible identified by comparison to mass spectral databases.
- Target component analysis may be performed to compare specific data sets.
- Individual datapoints from the chromatogram will be compared between groups using ANOVA and ROC analysis, to identify potentially discriminating compounds

Summary

A sampling approach used on skin has been shown in previous work to be robust and reliable for VOC profiles from all but the most delicate structures of human skin. The analytical utility of this technique and its compatibility with existing instrumentation has been demonstrated on skin and this study aims to extend this simple methodology to further studies.

Our non-invasive methodology aims to provide next generation *in-vivo* VOC sampling techniques for the discovery of endogenous VOC chemistry. If successful this sampling approach will then be applied to phenotypically distinct groups of people in prospecting for chemical markers of health and vitality.

Analysis is performed using thermal desorption gas chromatography mass spectrometry (TD-GC-MS) yielding complex profiles that present a significant data processing challenge. A retention index based on Kováts system is employed to generate component libraries and facilitate sample comparison, combining this with mass spectral deconvolution provides part of a comprehensive data processing strategy for identification of biomarkers and yet unidentified components in VOC profiles.

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Appendix 1.c: Participant information sheet

Skin Analysis in <Study Title>>

Part 1

Invitation to participate

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish. This part (Part 1) tells you the purpose of this study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study.

Please ask us if there is anything that is not clear, or if you would like more information. Please take time to decide whether or not you wish to take part.

*What is **the** purpose of this study?*

We are testing a new way of detecting changes in the human body by monitoring tiny amounts of chemical substances on skin. These substances arise from all the processes and reactions that occur in the body and we think that analysis of these compounds may be able to give us information about health and well-being of individuals.

Why have I been invited?

You will have been invited because you have << specific inclusion criteria goes here>> and this makes you part of the group of people we are particularly interested in seeing.

Do I have to take part?

It is up to you to decide.

We will describe the study and go through this information sheet, which we will then give to you. You will have the opportunity to ask questions of the researchers and be given time to consider whether you wish to participate. We will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, and you do not have to give us a reason.

What will happen to me if I take part?

If you agree to take part we will arrange for you to visit our laboratory for a single visit lasting about an hour. During this visit we will first ask you a series of questions relating to factors that can influence the chemical substances on your skin. These will include questions about food, drink, medications recently consumed, and smoking habits. We will also ask you some questions about personal care including toiletries, perfumes and personal care products. We will also ask you for information about diet and digestion, and finally we will ask for some information about <Inclusion criteria specified here>.

If you would like a chaperone of the same sex to be available during your study visit this will be arranged for you.

For the same reasons, you may be asked to refrain from eating, drinking (other than unflavoured water) and smoking for an agreed time period prior to the study visit.

After we have collected this information, we will then begin by taking a single sample. This involves placing a small section of an inert membrane on the surface of your skin. The membrane will be held by a generic dressing usually used for first aid. You will be asked to sit comfortably while compounds from the surface of your skin absorb onto the membrane, each sample will take up to a maximum of 30 minutes and we will ask for up to 5 samples, however samples are often taken simultaneously. You are under no obligation to complete the full 30 minutes or give more than one sample and you can stop that study at any time with no consequences.

What will I have to do?

We need you to not smoke, eat or drink (although you are permitted to drink unflavoured water) for an agreed time period prior to the study visit.

We also ask you not to use perfumed products (which include not only perfume, but also deodorants, after-shaves), any facial products (such as make-up or creams (scented or un-scented)), for an agreed time period (usually a few hours) prior to your visit.

What are the possible disadvantages and risks of taking part?

We do not anticipate any risk from taking part in this study. There is the possibility of allergic reaction to the silicone sampling device however this is very rare and as silicone is used in cookware, baby's bottles and many medical devices including incubators, catheters and dressings we think it highly unlikely that you will experience any adverse effects. We will always be with you during this process and you will be in complete control of the sampling and be able to stop it quickly and easily whenever you chose.

What are the possible benefits of taking part?

There will be no direct benefits to you from taking part in this research. The aim is to develop a new test that may be able to provide objective information about state of health and well-being.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. You may withdraw from the study at any time; either before or during the study. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

Part 2

What will happen if I don't want to carry on with the study?

You are under no obligation to participate in this study and you may withdraw from the whole study, or contribute only to a part of the study (for example you may not wish to give a sample, or answer the questionnaire, or *vice versa*), in which case we will use only the sample(s) and answers you agreed to provide.

We will ensure that you are able to stop the sampling procedure at any time quickly and easily should you want to.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (contact number 0150 922 2549). You may ask a researcher to clarify anything about the study at any time, both before and during sampling. If you remain unhappy and wish to complain formally, you can do this through Loughborough University Complaints Procedure. Details can be obtained from the University.

In the event that something does go wrong and you are harmed during the research due to someone's negligence then you may have grounds for a legal action for compensation against Loughborough University but you may have to pay your legal costs.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential, and any information about you which leaves the Laboratory will have your name and address removed so that you cannot be recognised.

What will happen to the samples I give?

The samples are collected on the patches of membrane that are placed on your skin, these are then removed and placed in small tubes that are sealed and will be processed at Department of Chemistry at Loughborough University. Your name will be removed, and the samples identified only with a code. No genetic testing will be performed on any of your samples.

What will happen to the results of the research study?

It is intended to publish the results of this study in a scientific journal. Data generated will be kept at the Department of Chemistry at Loughborough University. Your name and address will not be kept with the data although a record of your participation will be maintained by the

principle investigator. The measurement data will only be made available to people who are directly involved with the study.

Who is organising and funding the research?

This study is a collaboration between Loughborough University and Unilever PLC.

Who has reviewed the study?

This study has been reviewed and been given a favourable opinion by Loughborough University Ethics Committee to protect your safety, rights, wellbeing and dignity.

Who should I contact for further information?

Prof. C. L. Paul Thomas
Professor of Analytical Science
Department of Chemistry
Loughborough University
LE11 3TU
Tel: 0150 922 2549

Appendix 1.d

CONSENT FORM for Skin analysis in <Study Title>> .

Mr/Mrs/Miss/Ms/Dr.....
Name.....
Age.....
Contact details.....

Trial Supervisor
Prof. Paul Thomas, Professor Analytical Science, Department of Chemistry, Loughborough University, LE11 3TU
Tel. No. 0150 922 2549
Before participating in our study we request that you complete this consent form.
Please circle the appropriate response.

I have been given a copy of the information sheet Skin Analysis in <Study Title>>	Yes	No
I understand that my participation in this study is voluntary and that I am under no obligation to take part.	Yes	No
I have read and understood the information sheet	Yes	No
I have had the opportunity to ask questions and I understand the answers I have been given.	Yes	No
I understand that I may withdraw from the study at any time	Yes	No
I have been shown how to stop the sampling procedure if I want to.	Yes	No
I consent to participate in this study	Yes	No

PLEASE SIGN HERE

Date
Many thanks for your help.

Researcher use

Participant identifier:

Appendix 1.e:

Health screen questionnaire

Female Health Screen Questionnaire For skin analysis in <Study Title>> .

Participant Identifier.....

Trial Supervisor
Prof. Paul Thomas, Professor Analytical Science, Department of Chemistry, Loughborough University, LE11 3TU
Tel. No. 0150 922 2549
Before participating in our study we would like you to complete this health screen questionnaire, you are not obligated to answer any of the questions if you feel uncomfortable about doing so.

Please circle the appropriate response.

Do you have any allergies that you are aware of?	Yes	No
Do you have any specific dietary preferences? (eg, vegan, vegetarian)	Yes	No
Are you taking or using any medicines, pills, tablets, ointments, injections or any other drug, either from your doctor or on your own accord?	Yes	No
Have you experienced nausea, sickness or dizziness in the last week?	Yes	No
Do you have sensitive skin?	Yes	No
Is there anything else concerning your health, you think we should know about?	Yes	No

If you answered 'Yes' to any of the above could you please provide further information here:

.....

.....

.....

Please inform us of any changes in the above before the study starts.

For Female Participants only

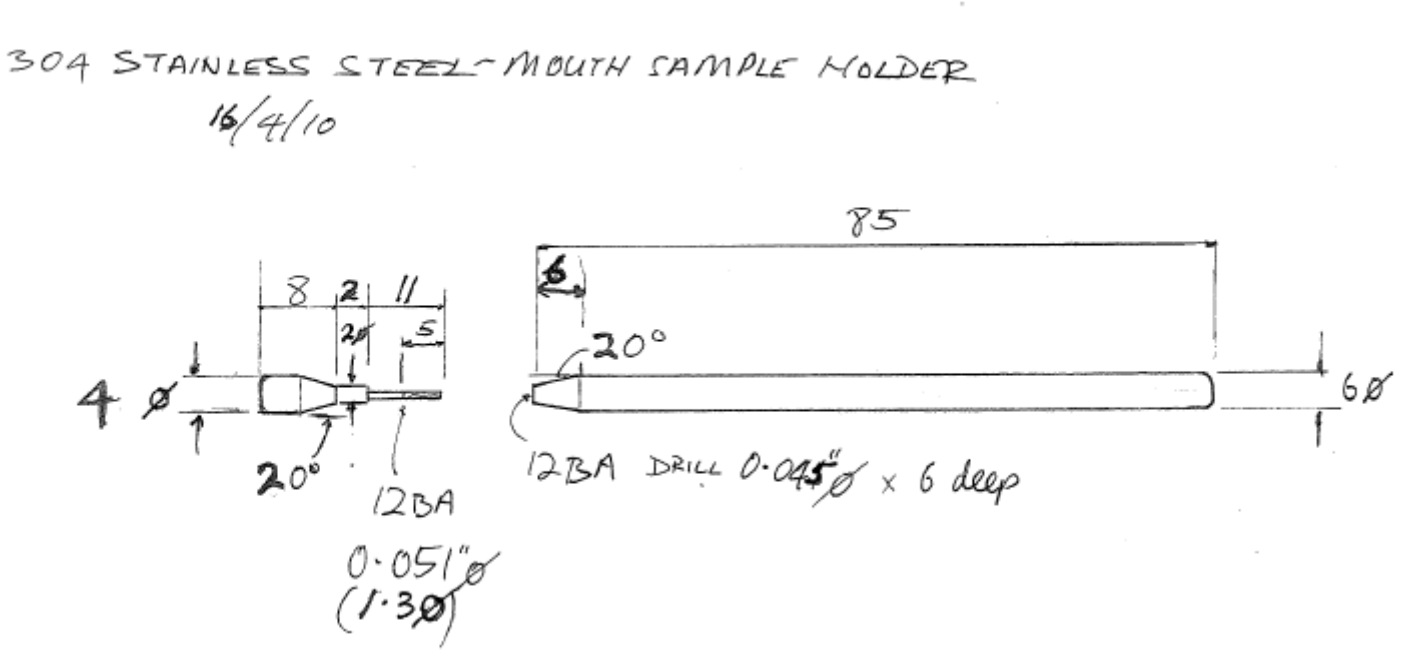
Are your periods normal/regular?	Yes	No
Are you on the contraceptive 'Pill'?	Yes	No
Do you use any other form of contraception that may affect your menstrual cycle? (Implants, injections, IUD etc)	Yes	No
Could you be pregnant?	Yes	No

To the best of my knowledge this information is correct. I confirm that I have read and understood the information sheet for the above study and have had the opportunity to ask questions.

PLEASE SIGN HERE.....
Date.....
Many thanks for your help.

APPENDIX 2: THE ORIGINAL DESIGN OF THE SALIVA SAMPLER

The original design for the saliva sampler in Figure 3.4, reproduced here with the permission of Peter Carroll, Unilever PLC.



APPENDIX 3: COMPUTATION OF PHYSIOLOGICAL STRESS RESPONSE SCORES

This excerpt is taken from M. A. Turner *et. al.* 'The effect of a Paced Auditory Serial Addition Task (PASAT) intervention on the profile of volatile organic compounds in human breath: A Pilot Study' [63] J. Breath Res. 7 (2013) 017102

PASAT response score

The participants' individual cardiovascular responses to the PASAT and neutral interventions were scored on the basis of observed changes in their systolic blood pressure and heart rate. The average of the three initial baseline observations were used to give BP and HR start values ($P_{\text{start }S}$ and HR_{start}). The maximum observed values of the four readings taken during the intervention periods ($P_{\text{max }S}$ and HR_{max}) were assigned the

points of maximum 'stress'. A PASAT response score (PSR) may be derived for the PASAT (PSR_S) and neutral interventions (PSR_N) by:

$$PSR = \frac{\Delta P_S}{P_S^{Start}} \times \frac{\Delta HR}{HR^{Start}} \times 1000$$

Where

$$\Delta P_S = P_S^{Max} - P_S^{Start}$$

and

$$\Delta HR = HR^{Max} - HR^{Start}$$

The PSR sought to emphasize the relative change in systolic blood pressure that occurred during the intervention (ΔP_S) compared to baseline observations (P_S^{Start}). Heart rate was also included in the same manner providing a ratio between the overall change $(\_HR)$ and the baseline values. The PSR score was framed to give higher score for those individuals showing the largest cardiovascular responses under the interventions. One male participant did not exhibit a stress response (PSR_S = 0.9, PSR_N = 5.3). The average response for the remaining 21 participants was a PSR_S of 64.5 compared to PSR_N score of 4.5. The median PSR score during the neutral intervention was 3.5 compared to 42.8 for the PASAT intervention. Male and female responses were not statistically different; female PSR scores showed a greater range of responses during the PASAT intervention (PSR = 12 to 233), while it was the male participants who had the greatest variability (PSR = 0.1 to 18.6) in the neutral state. Overall, the cardiovascular responses summarized in figure 1 and by the PSR scores are consistent with the participants experiencing psychological stress from the PASAT intervention.

APPENDIX 4: PHYSICAL STRESS STUDY VISIT TIMELINE

Introduction and Briefing	0 - 30 min		30 - 60 min	65 - 90 min	90 - 120 min	End Phase
	Phase A (Baseline Measurements)		Phase B (Physical stress)	Phase C (Recovery phase 1)	Phase D (Recovery phase 2)	
Outside chamber and changing facilities	Scales in lab and bathroom	Sampling Area	Bicycle Ergometer	Sampling area		
Change	Weight & Height	Urine 1	Explaination of sampling during exercise	Skin patches ON (3)	Heart rate monitor OFF	
Questions	Allow subject to drink water/rinse mouth (100ml)	Skin patches ON (1)	Help subject onto bicycle ergometer	Saliva (hormones & metals) 2	Skin conductivity meter off	
Consent form		Heart rate monitor ON	Skin patches ON (2)	Saliva (volatiles) 2	Skin patches OFF (4)	
Information compliance		Rinse forehead and place skin patches ON (PDMS & cotton)	Skin conductivity meter manual time stamp 2	Breath 2 (Using breath mask)	Saliva (volatiles) 3	
Health Screen Questionnaire		Skin conductivity meter ON (automatic time stamp)	10 x DMS readings every 3 minutes (at 2, 5, 8, 11, 14, 17, 20, 23, 26, 29 minutes into exercise)	Blood 2	Saliva (hormones & metals) 3	
		Saliva (hormones & metals) 1	Please note 0 and 30 minutes are the start and end of the exercise, respectively	Allow subject to drink water (100 ml)	Skin patches ON (4)	
		Saliva (volatiles) 1	6 x heart rate readings every 5 minutes (0, 5, 10, 15, 20, 25, 30 minutes in exercise).	Urine 2	Breath 3 (using breath mask)	
		Breath 1 (Using breath mask)	Skin patches OFF (2)	Help subject off bicycle ergometer	Blood 3	
		Blood 1		Skin patches OFF (3)	Allow subject to drink water (100 ml)	
		Skin patches OFF (1)			Urine 3	
		Skin conductivity meter manual time stamp 1			Heart rate monitor OFF	

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