Discovery of Stress Biomarkers in Biological Matrices using Novel Sample Collection Techniques, Inorganic and Organic Mass Spectrometry and Multivariate Analysis

Pareen Patel

A Doctoral Thesis

Submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of Loughborough University

November 2013

Abstract

New methodologies for the collection and analysis of biological samples from psychological, physical and emotional stress are described. Currently, there is little research relating to the elemental, VOC and small molecule changes in biological samples as a consequence of stress on the human body, with much of the current research indicating physical symptoms.

This research sought to measure chemical changes in three different categories of stress. The first uses an existing PASAT intervention to induce psychological stress and a further two new methodologies using exercise to induce physical stress and a trapped human in a simulation of a collapsed building to induce emotion stress. Psychological, physical and emotional stress elemental profiles are compared against their respective chemical baseline profiles. Skin samples are collected from the foreheads of participants who endured emotional stress while drool saliva, urine, plasma and forehead skin samples were obtained from physically stressed participants. Furthermore, drool saliva is also obtained from the individual who experienced emotional stress. Multi elemental analysis of biological samples is performed using sector field inductively coupled plasma mass spectrometry (ICP-MS). Elemental data acquired from ICP-MS enabled the construction of individual biological data sets for examination by multivariate analysis to define potential biomarkers of stress.

Three sets of saliva data obtained from the cohort of physically stressed participants are analysed using ICP-MS, thermal desorption gas chromatography mass spectrometry and ultra-performance liquid chromatography ion mobility mass spectrometry producing elemental, VOC and small molecule data, respectively. Stress biomarkers in saliva are discovered using multivariate analysis.

A skin sampling device is designed, constructed and tested on cohorts of healthy participants. The analysis of skin samples using ICP-MS and electrospray ionisation mass spectrometry exhibits successful extraction of elements, VOCs, semi-VOCs and small molecules from localised regions of the human body. Sixty of ninety six candidate skin biomarkers are identified using nano electrospray ionisation mass spectrometry.

Acknowledgements

This research was funded by a Loughborough University Development Fund PhD studentship that was awarded to the Research School of Health and Life Sciences.

I would like to acknowledge my supervisors Dr Helen Reid and Prof Paul Thomas for all their support and guidance. I am very grateful to have been presented this research opportunity and be a part of their research groups. This great research opportunity would not have existed had it not been for Helen securing the project.

There were many people who collaborated on the projects presented in this thesis, but a special mention to Matthew Turner, Helen Martin and Aditya Malkar for their dedication into making us into a team who would always help each other. Acknowledgments must also be mentioned to Dr Corrinne Burns and Dr James Reynolds for their huge contributions on the skin sampling device. To Dr Phillip Watson for blood sampling and Prof Ronald Maughan for allowing the team to use the facilities in the SSEHS during the physical stress study, Dr Stephan Bandelow and Dr Louisa Edwards for presenting the PASAT for the psychological stress study, to Dr Svetlana Riazanskaia for the opportunity to work on the skin sampling device and Peter Carroll for engineering it. To Prof Barry Sharp for allowing me to work in his laboratory and Prof Colin Creaser for his guidance.

My gratitude goes out to all my colleagues working at the Centre for Analytical Sciences at Loughborough University's Department of Chemistry especially to Dr David Douglas for his everlasting patience with my constant questioning regarding the ICP-MS. But also to Dr Aref Zayed, Dr Cris Guallar-Hoyes, Dr Dhinesh Asogan and Sarah Taylor. A mention must also go to Thermo Scientific engineer David Hinds, for his patience and advice in maintaining the ICP.

The most important thank you goes to Sarah-Jane Dudney. Her love and support has given me the life I needed away from work. "Jane" ensured I found time to relax away from work and we have been on some truly great holidays that some people can only dream of. Moving on with the next chapter of our lives is something we've longed for and now it's finally happening. I hope we create many more memories along the way.

Completing this research could not have been possible without my family. My amazing mother, Pragna, for her unconditional love and support away from my research. My brother Atish always giving me that reality check, one which *he* always seemed to think I needed. It's a shame you're now living in Perth. Of the many good things to come out of the few years studying for my PhD this is one of the most important to me: I've grown a lot closer to my sister Maya-Krupa. And the rest of my family; Nayan, Peter, Mary, Rob, Owen, James, Helen, Amit and Sarah L.

Hard work, determination and graft. It's because of all the people I've come across in my life I find myself in the position I am today. My final gratitude must go to the influences of my close friends from my time at Sheffield University, without them there is absolutely no way I would be writing this thesis: Dr Andrew Morse, Dr Mark Moxey, Sean Staunton, Masim Sayed, Tom Nixon and Dr James Newby.

Table of Contents

List of Table

Table of Figures

Keywords

Stress; Data stitching; ICP-MS; ESI-MS; Biomarkers; Multivariate analysis; The Liquid Skin Pen; Elemental analysis;

Glossary of Terms

μ	Arithmetic mean of a data set
σ	Standard deviation of a data set
$\mu g.mL^{-1}$	Micrograms per litre (parts per million)
A	Number of components
ANOVA	Analysis of variance
Axilla	Skin sample site at the human arm pit (AP)
BP	Blood-pressure
$C_2H_4O_2$ (aq)	Acetic acid (aqueous)
DI H_2O	Deionised water
Dorsal	Skin sample site at the top of a human foot (TF)
DMS	Differential mobility spectrometry
EDC	Enhanced duty cycle
EDC	Enhanced duty cycle
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
ESI-MS	Electrospray ionisation mass spectrometry
ES-	Negative ion mode
ES+	Positive ion mode
EtOH (aq)	50% ethanol (aqueous)
FB	Field blank
H_0	Null hypothesis
H_2O	Water
HDMS	High definition mass spectrometry
HNO_3	Nitric acid
HR	Heart rate
ICP-MS	Direct injection sector field inductively coupled plasma mass spectrometry
$L.min^{-1}$	Litre per minute
LOD	Limit of detection
LOQ	Limit of quantification
LSP	Liquid skin pen
$ml.min^{-1}$	Millilitre per minute
ml	Millilitre
MVA	Multivariate analysis
m/z	Mass-to-charge ratio
N	Number of observations in the model

nESI-MS	nano-Electrospray ionisation mass spectrometry
$ng.mL^{-1}$	nanograms per litre (parts per billion)
$NH_{3\ (aq)}$	Ammonia (aqueous)
P	Probability
PASAT	Paced auditory serial addition task
ParN	Pareto distribution (Normalised)
PB	Pen Blank
PC	Principal Component
PCA	Principal Components Analysis
PEEK	Polyether ether ketone
$pg.mL^{-1}$	picograms per litre (parts per trillion)
Plantar	Skin sample site at the bottom of a human foot (BF)
PLS-DA	Projection to Latent structures Discriminant Analysis
Q^2	Cumulative overall cross validated R^2X
R^2X	Cumulative x-variation modelled after A components
rpm	Revolutions per minute
RSD	Relative standard deviation
SRM	Standard reference material
S_S	Sampling solvent
Semi-VOC	Semi volatile organic compounds
TMAH	Tetramethylammonium hydroxide
TOF	Time of flight
VOC	Volatile organic compounds
Volar	Skin sample site at the human anterior elbow (AE)
W	Watt
$W.kg^{-1}$	Watts per kilograms of total body weight

1 Introduction: The potential to diagnose human vitality and wellbeing from biological fluids

1.1 Research Objective

The first aim of this research was to find detectable differences in trace element profiles, from biological fluids in the human body, in individuals who possess different levels of health and lead different lifestyles and also differences in the same individual between different states e.g. stressed/unstressed physically or mentally. These profiles can be collected using fluids such as blood, saliva, sweat and urine (both invasive and non-invasive sampling techniques) and any differences in the profiles can be identified using a variety of techniques including inductively coupled plasma mass spectrometry (ICP-MS).

Elemental data obtained from a variety of biological fluids could be integrated into one large data set and examined using multivariate analysis (MVA) by constructing statistical models. Furthermore, elemental and molecular data obtained using a range of mass spectrometry techniques to analyse the same biological fluid could also be combined and examined by MVA with the intention of creating a holistic picture of the effects of lifestyle and stress on the chemistry of the human body.

The final aim was the design and development of a non-invasive skin sampling device targeting several human body sites. The long term goal of this device was to enable online analysis of a real time stress state of a person that can be identified in the skin from various sites of the human body.

Details of the chemicals used for all the experiments and studies performed in this work can be found in this chapter along with the information regarding ethics and participant information for studies involving human subjects. Additionally, information regarding the mass spectrometry techniques used to analyse samples collected from this work, including inductively coupled plasma mass spectrometry, electrospray ionisation mass spectrometry and nano-electrospray ionisation mass spectrometry, are detailed in greater depth in this chapter. A brief overview is also provided of the statistical analysis used for all analysed data in this research. This includes an attempt at multivariate analysis to investigate changes in sample data obtained from human studies in combination with commonly used statistics to identify whether differences exist between two or more sample sets.

1.2 Stress

The term stress was first applied by the endocrinologist Han Seyle in the 1930's. It is a response to particular events in the way our body prepares itself to face a difficult situation with strength, focus and heightened alertness that has been defined as the failure of an organism to respond following physical or emotional threats.[1] Stress is defined by psychologists as how the body reacts to a stressor (a stimulus that causes stress), real or imagined.[2]

Seyle defined three reaction stages in which the body responds to stress, known as the general adaptation syndrome. The first of these stages, the alarm stage, is when the body's natural response is a state of alarm when the stressor has been identified. The nervous system responds by releasing an excess of stress hormones, including cortisol and adrenaline to prepare for a fight or flight response. These hormones rouse the body for emergency action and if the stressor continues the next stage, resistance, is entered. Here the body begins to try and cope with the stress. However, the adaptation of the body to the strains of the environment cannot be maintained for an indefinite period and resources being used are gradually depleted. It is at this point the third and final stage, exhaustion, may be entered. The body is unable to maintain normal function due to the majority of its resources nearing depletion, and if exhaustion is prolonged, the danger of long term damage to the body may be the consequence. Should this stage persist, a number of illnesses may be observed including depression, trouble with the digestive system, ulcers, cardiovascular problems and mental illnesses.[3]

It is anticipated that by observing composition changes in a number of biological fluids following stress, future medicines can inhibit these composition changes and thus suppress the onset of chronic stress. The primary function of sweat is thermoregulation[4] but events such as stress induce spurts of sweating waves.[5, 6] Emotional sweating is commonly measured on the glabrous surface of the palms.[7–10, 6] However, it isn't always possible to assess emotional stress using the palms when manual work is undertaken using both hands. Emotional sweat is often observed on the forehead but few studies have researched it.

Sweat has been described as a good indicator by Shimoda *et al*. who measured mental strain by forehead sweat in students. They found that bursts of forehead sweating waves developed in 78% of the participants when they encountered difficult parts of the task. Students who failed to show any increase in sweat in their first attempt at the task did show sweating in their second attempt. Excretion of thermal sweating increases with ambient and

body temperature[4] so during the experiment the ambient temperature was monitored between 21-24 °C in order to minimise heat exchange between the environment and the participants skin. By excluding the environmental factor, their findings show a correlation between increased forehead sweat and emotional stress experienced.[11] Kamei *et al*. showed that when sweating is induced by physical or emotional stimuli, the behaviour of active sweat glands and sweat rate from the palm and forehead can be used to estimate the degree of emotional stress on humans. A periodical damped oscillation of sweating in both the sample sites was observed when a loud sound was produced behind the participant's head. If the participant was given a dose of caffeine the degree of sweating became 1.5-2 times greater compared to without the dose.[12]

Skin conductance shows the emotional state, as reflected in changes in the sympathetic nervous system. Storm measured behavioural state and skin conductance changes from the medial side of the right foot of premature infants and found that emotional sweating was observed in infants from 29 weeks gestational age and more than 10 days old.[13] In some cases, prolonged stress can be dangerous in certain situations making it necessary to collect feedback to control it.

Galvanic skin response (GSR) is a term coined after Luigi Galvani after he took an interest in the field of medical electricity. Measurement of GSR is effective when the sympathetic nervous system is active, especially when an individual is anxious, measuring a change in the electrical conductivity of the skin caused by an increase in sweat glands[14]. A GSR device sends a small current through the electrodes, measuring the conductance of skin, producing a continuous signal that varies with the emotional state of the subject. First documented in the early 1900's to explore the GSR for psychiatric evaluation and therapy[15], most applications since have focussed on utilizing the GSR to gauge emotional states such as anxiety and agitation.[14]

Villarejo *et al*. built their own stress sensor based on GSR, in a study where participants completed different tasks requiring a certain degree of effort, such as breathing deeply or mathematical operations, and found they were able to detect different states of each user with a success rate of 76.56%.[16] Nourbakhsh *et al*. used GSR for cognitive load measurement in arithmetic and reading tasks and found strong correlation between GSR and difficulty of the task being performed.[17] The GSR is known today as a component of a polygraph and as with cardiovascular measurements, an increase in GSR can indicate anxiety or nervousness when questions are being asked.

Saliva is produced by three major salivary glands (parotid, submandibular and sublingual) and a number of minor salivary glands. Glands consist of specialised epithelial cells with defined functional and morphological end pieces (acini and intercalated ducts) as well as a system of glandular ducts. Secretion is thought to be a two stage process. Firstly an aqueous plasma-like fluid is secreted by the acinar cells and secondly the fluid is modified during transport through the water impermeable ductal cell system. The autonomous nervous system controls the secretion via signal transduction systems that couple receptor stimulation to ion transport and protein secretory mechanisms.[18]

A number of pathologies of oral cavity have been related with stress including conditions such as ulcerations, periodontal disease, dental cavities, acute necrotizing ulcerative gingivitis and upper respiratory functions. Relationships have been discovered between these oral cavity conditions and stress based on epidemiological research, clinical observations and experiments with animals.[19–24] Given the importance of the protective function of saliva it is surprising that little research has been carried out on possible links between oral disease and psychological function considering saliva possesses a multiplicity of defence systems against toxins, viruses, fungi and bacteria.[25] Acute psychological stress influences both salivary composition and salivary function. It has been shown to significantly increase salivary protein concentration, amylase/protein ratio, α-amylase activity and s-IgA output.[26]

Skin is an organ worn on the outside of the body consisting of two main layers, the epidermis and dermis, with the organ having a primary physiological function of a barrier between the body and the external environment, protecting the body against excessive water loss[27] and pathogens.[28] Other functions of the skin include heat regulation and insulation against the environment,[29] control of evaporation, excretion,[30] synthesis of vitamin D[31] and sensations.[32] The outer layer, the epidermis, contains several layers – the stratum corneum, granular cell layer, spinous cell layer and basal cell layer. Below the epidermis is the layer called the dermis, which contains a variable amount of fat, collagen and elastin fibres its strength and flexibility.[33]

Skin sampling can be minimally invasive exposing subjects to less discomfort and reducing the risk of complications. A number of minimally invasive methods to sample skin already exist to monitor bodily functions and responses, monitor diseases and assess drug levels.

A great deal of research time is presently being devoted to the analysis of skin-secreted compounds, with the ultimate objective of developing a system of odour-based diagnostics.

Katona *et al.* used desorption electrospray ionisation to rinse the skin and found signals of protonated urea, alkali adducts of urea and protonated creatinine in positive ion mode, and a variety of carboxylic acids, such as pyruvic acid, palmitic acid and stearic acid, in negative ion mode.[34] Riazanskaia *et al.* collected sweat from human forearms and abdomens using a polydimethylsilicone membrane successfully recovering a number of fatty acids including isobutyric, butanoic, 3-methylbutanoic, pentenoic and heptanoic acids.[35] Lidén *et al.* used cellulose wipes, containing 1% nitric acid, to sample contact allergens from the arms and palms on skin. Using this technique they recovered over 90% of all selected elements that included nickel, chromium and cobalt.[36] Tape-stripping has been a popular method to evaluate processes of human skin. Stinchcomb *et al.* researched the chemical uptake into human stratum corneum from volatile and non-volatile solvents and found that increasing the duration of exposure of a modelled compound increased the kinetics of uptake using a volatile solvent when compared to the theoretical diffusion rate.[37]

1.3 Inductively Coupled Plasma-Mass Spectrometry

Due to its high resolving power, excellent detection capabilities, large dynamic range and very high sensitivity, the double-focussing magnetic sector technology was employed throughout the presented work. Ions are initially produced in the plasma after which they are accelerated to a few kilovolts in a vacuum. They are then focussed through an entrance slit and enter the mass analyser, where ion separation takes place. Following separation the ions pass through an exit slit and strike a detector producing an electrical signal that is transferred to a computer.

ICP-MS is an analytical technique capable of detecting metals and numerous non-metals at concentrations as low as parts per quadrillion ($pg.L^{-1}$). ICP-MS offers further major benefits as well as high detection power such as wide linear calibration range, greater speed, precision, very low limits of detection for most elements and high sample throughput for clinical assays (Table 1.1). Previously, other atomic spectrometric techniques, such as atomic absorption spectrometry (AAS), had been the commonly used analytical method for screening studies and everyday clinical assays.[38–40]

ICP-MS does have its limitations, especially with regard to biological applications. Spectral and non-spectral interferences are the major limitations the ICP-MS suffers and they are extremely serious in the analysis of biological materials.[41, 42] These spectral interferences are a result of atomic or molecular ions having similar nominal masses as those of the analyte of interest. There are also interferences from plasma gases and solvent. Major components of biological matrices are renowned to form a variety of possible polyatomic ions that can overlap several isotopes.[41, 42]

High levels of organic and inorganic salts in urine, blood and serum samples will often lead to isobaric overlap and matrix interferences as proved by Hsiung *et al*[43], Barany *et al*[44] and Nixon and Moyer[45]. In these studies, concentrations of elements such as As, Cd, Pb, Tl, Cu, Zn, Co, Ni and Se in blood and urine sample were determined using a quadrupole ICP-MS instrument. At the heart of the discussion for these studies, were problems caused by spectral interferences predominantly for those elements with an atomic mass lower than 80 u. These spectral interferences are predominantly a result of a relatively high concentration of organic species in these biological applications. An example of this interference can be seen in the determination of Cu in urine samples using quadrupole ICP-MS, studied by Hsiung *et al*.[43] With argon being used as the flow gas, ^{63}Cu was found to suffer from major

spectral overlap from $^{40}Ar^{23}Na$. As a result, ^{65}Cu was selected due to a low possibility of interferences being present for this isotope.

It is believed that matrix interferences is a result of ionisation energy, sample matrix, plasma operating conditions and mass of the internal standard relative to the analyte, as well as other factors.[46]

A great deal of polyatomic interferences typically encountered in biological applications can be overcome by employing the ICP-MS in a high resolution setting, where the result of low background and high sensitivity can produce extremely low detection limits.[47–52] High resolution inductively coupled plasma (HR ICP-MS) has been used in clinical applications to determine precious metals in blood,[53, 54] uranium in urine,[55] calcium isotope ratios in urine[56] and trace elements in serum.[40, 49, 57] There are numerous ways used to correct the signal for non-spectral interferences which include internal standardisation, matrix-matched standards and standard additions techniques.[41, 58, 59] As well as these, ICP-MS is susceptible to contaminations from reagents and glassware due to its high sensitivity.

Bio monitoring of trace elements in body fluids is generally performed to investigate the concentration of essential traces or to determine toxic substances. Bio monitoring of toxic or carcinogenic elements is important as it can lead to evidence of exposure of hazardous substances from places such as industry or the environment. Toxic or carcinogenic elements, such as Be, Pb, Pt, Cd, U, are relevant in occupational and environmental medicine. For example, Be and its compounds cause a chronic disease that primarily affects the lungs[60]. High concentrations of Pb can cause high blood pressure, anaemia or kidney injury[61]. Also, Pt, Tl, Cr, Cu, Sn affect the kidneys if at higher concentrations whilst U compounds can damage the liver and cause internal haemorrhaging and Sn present in organic compounds have high toxicity.[62–64]

Table 1.1. Summary of elemental analysis techniques

		Flame AAS	GFAAS	ICP-AES	ICP-MS
Detection limits		Very good for some elements	Excellent for some elements	Very good for most elements	Excellent for most elements
Sample throughput		10-15 secs per elements	3-4 mins per element	1-60 elements/minute	All elements <1 minute
Dynamic range		10^3	10^2	10^6	10^8
Precision	Short term	0.1-1.0%	0.5-5%	0.1-2%	0.5-2%
	Long term	2-beam 1-2% 1-beam < 10%	1-10% (tube lifetime)	1-5%	2-4%
Interferences	Spectral	Very few	Very few	Many	Few
	Chemical (matrix)	Many	Very many	Very few	Some
	Physical (matrix)	Some	Very few	Very few	Some
Dissolved solids in solution		0.5-5%	> 20% (slurries)	0-20%	0.1-0.4%
Elements applicable to		68+	50+	73	82
Sample volumes required		Large	Very small	Medium	Very small to medium
Semi-quantitative analysis		No	No	Yes	Yes
Isotopic analysis		No	No	No	Yes
Ease of use		Very easy	Moderately easy	Easy	Moderately easy
Method development		Easy	Difficult	Moderately easy	Difficult
Unattended operation		No	Yes	Yes	Yes
Capital costs		Low	Medium to high	High	Very high
Running costs		Low	Medium	High	High
Cost per elemental analysis					
High volume - few elements		Low	High	Medium	Medium
High volume - many elements		Medium	High	Low-Medium	Low-Medium

Typically there are four major parts to the ICP-MS; the sample introduction system; the plasma; the interface region; and the mass analyser and detector (Figure 1.1).

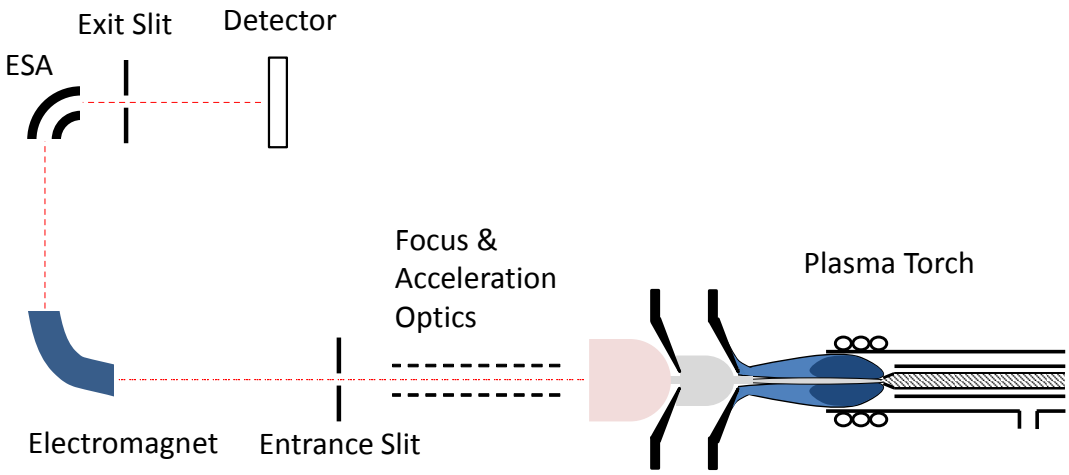


Figure 1.1. Schematic diagram of a SF-ICP-MS system in a reverse Nier-Johnson geometry

1.3.1 Sample introduction

Most ICP-MS applications involve the analysis of liquid although the technique has been adapted to handle solids. A number of ways are possible to introduce liquid into the ICP-MS but the aim of all is to generate a fine aerosol of sample in order for it to be efficiently ionised in the plasma discharge. However, only 1-2% of the sample finds its way into the plasma making the sample introduction the weak point in this technique. There are two separate stages for introducing the liquid into the analytical plasma. The first is aerosol generation using a nebuliser, and the second is droplet selection from the use of a spray chamber because the plasma discharge is inefficient at dissociating large droplets. The sample is pumped at a low pressure but high speed of approximately 1 $ml.min^{-1}$ using a peristaltic pump, which ensures a constant flow of liquid regardless of viscosities between samples, standards and blanks. The liquid is then broken up into a fine aerosol by the pneumatic action of the gas flow smashing the liquid into small droplets when the sample enters the nebuliser. It must be noted that although this is the most common approach, some nebulisers (concentric design) rely on the natural Venturi effect of positive pressure of the nebuliser gas to suck the sample through the tubing. Concentric nebulisers have excellent sensitivity and stability but their small orifices can be plagued by blockage problems. Other nebulisers have proved useful for different applications, such as the cross flow nebuliser that is used for heavier matrixes and small undissolved matter or the micro flow nebuliser that is required for lower sample flow with high gas pressures used for limited samples. Whilst the primary function of the spray chamber is to only allow small droplets to

enter the plasma, its secondary purpose is to smooth out pulses that occur during the nebulisation process. Large droplets fall out of the spray chamber under gravity whilst the small droplets are transported to the injector. Spray chambers are used to increase sample efficiency and sensitivity whilst improving detection limits and selecting uniform droplets to decrease relative standard deviations. Whilst a number of spray chambers are available, the two commonly used ones are the Scott double pass and cyclonic spray chambers. The Scott double pass spray chamber selects small droplets and directs them to a central tube whilst the larger droplets fall out by gravity via a drain tube. This is achieved as the liquid in the drain tube is at positive pressure forcing the small drops between the central and outer tubes. The cyclonic spray chamber discriminates the size of droplets using centrifugal forces by vortex from the aerosol and the flow gas, commonly argon. Smaller droplets remain in the main stream of the aerosol whereas the larger droplets moved to the outer walls of the chambers and are forces out via a drain tube.

1.3.2 The Plasma

Prior to introduction into the mass analyser, the sample aerosol is ionised in the plasma. The plasma is formed by the introduction of an inert gas, commonly argon, into a torch which is made from quartz. Argon is used as it is cheap, very abundant (^{40}K decay) in the atmosphere and has a very high first ionisation potential ($Ar^+ + e^- \rightarrow Ar$ is more energetically favourable than $M^+ + e^- \rightarrow M$) therefore samples remain ionised. The torch consists of three concentric tubes of different diameters; the inner tube which carries the sample aerosol; the middle tube carrying a gas flow to shape the plasma; and the outer tube which carries the gas flow that cools the torch (Figure 1.2).

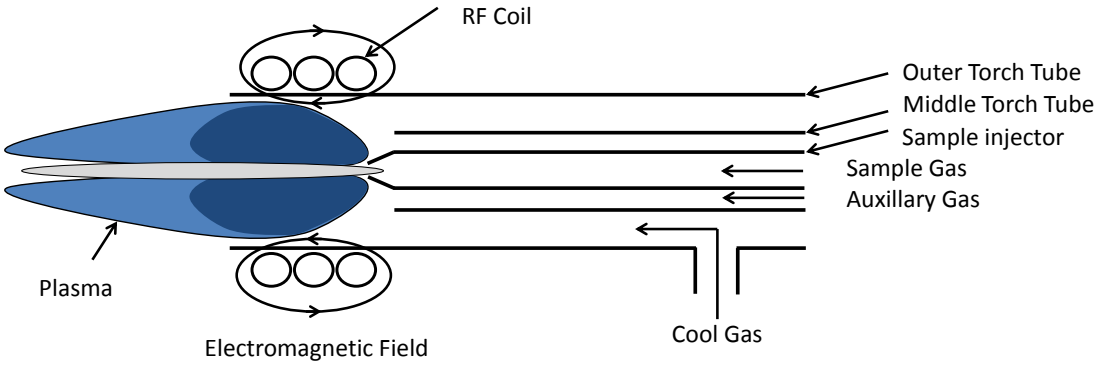


Figure 1.2. Schematic diagram of an ICP torch

Upon plasma initiation, the argon gas tangentially flows through the torch and a high spark voltage is applied, stripping electrons from argon atoms. A magnetic field, where these elections are captured and accelerated, is produced from a radio frequency (RF) coil that

surrounds the torch and typically operates at RF power of 750-1500 W depending on the type of sample. Collisions between these electrons and argon atoms cause ionisations producing chain reactions that eventually form an ICP discharge. The plasma is sustained when the rate of release of free electrons is proportional to the recombination of Ar atoms. When the sample exits the injector it is moving at such a velocity that it punches a hole through the centre of the plasma discharge where it then undergoes a number of physical changes due to the varying temperatures (6000–8000 K) of the plasma. The sample goes through desolvation, vaporisation, atomisation and finally ionisation starting in the preheating zone and continuing through the initial radiation zone before arriving in the analytical zone. During the desolvation step, water molecules are stripped away from the sample which then becomes a very small solid particle. As the sample travels further into the plasma these solid particles change into gaseous form and then ground state atoms after which ionisation is achieved by collisions of energetic argon electrons with these ground state atoms. As the ion emerges from the plasma it is directed into the interface region.

The movement of ions from the plasma to the mass spectrometer, via the interface region, is probably the most critical area of the ICP-MS. The role of the interface region is to transport ions efficiently, consistently and with electrical integrity from the plasma which is at atmospheric pressure (760 Torr), to the mass spectrometer analyser region which is at approximately 10^{-6} Torr.

1.3.3 The interface region

This region is maintained at a vacuum of ~3-4 Torr with a mechanical vacuum pump and is made up of two metallic cones with very small orifices (Figure 1.3). Ions generated from the plasma pass through the orifice (diameter of 0.8-1.2 mm) of the first cone (known as the sampler cone) after which they travel a short distance and through a second, much smaller, orifice (diameter of 0.4-0.8 mm) of the second cone (known as the skimmer cone). Typically these cones are made from nickel but can also be made from material such as platinum that are more resistant to corrosive liquid. Nickel cones are commonly used due to their low costs whereas aluminium cones are used when nickel is the element of interest. Also used are platinum cones, however, these are expensive so are used when oxygen is added for organic analysis. Due to the high temperature of the plasma this region is water cooled and made from material that dissipates heat easily such as aluminium or copper. The ion beam that emerges from the skimmer cone travels through the ion optics, where it is focussed, and guided into the mass separation device.

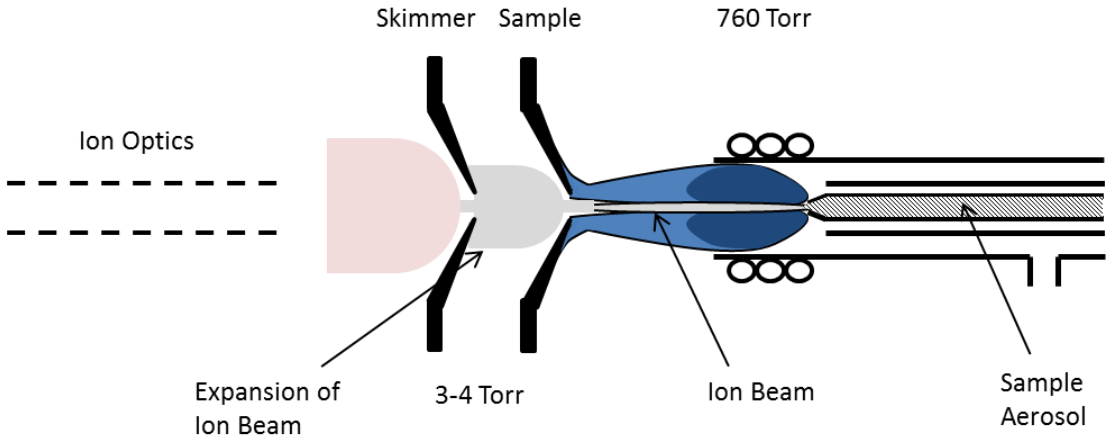


Figure 1.3. Schematic diagram of the interface region

1.3.4 The mass analyser and detector

The ion optics, mentioned above, consists of one or more electrostatically controlled lens components and are made up of a series of metallic plates, barrels, or cylinders that have high voltage on them. The role of the ion optics is to take ions from the hostile environment of the plasma at atmospheric pressure, via the interface cones, and direct them into the mass analyser, which is under high vacuum. It is essential the plasma is at zero potential ensuring that the magnitude and spread of ion energies are as low as possible and thus maintaining the composition and electrical integrity of the ion beam as it enters the ion optics.[65] An additional function of the ion optics is to prevent photons, neutral species and particulates entering the mass analyser and detector, which affect performance of the system by causing signal instability and increase background levels. There are two methods to reduce undesirable species getting to the mass spectrometer; the first is to place a grounded metal stop (disk) behind the skimmer cone blocking the undesirable species from travelling downstream but allowing the ion beam to move around it; and the second is to set the ion lens or mass analyser slightly off axis where positively charged ions are steered into the mass analyser by the lens system while the undesirable species are ejected out the beam. Some lens systems incorporate an extractions lens. This is placed after the skimmer cone and electrostatically pulls the ions from the interface regions improving the transmission and detection limits of low mass elements.

Various devices can be employed as the mass separation devices such as the quadrupole mass filter where a direct current (dc) field is placed on one pair of rods and a radio frequency (rf) field on the opposite pair, allowing ions of a selected mass to pass through the rods to the detector, while others are ejected from the quadrupole. Less frequently used devices, such as time of flight technology (TOF), can also be employed. Here, the kinetic

energy (E_K) of an ion is directly proportional to its mass (m) and velocity (v). If the population of ions, all having different masses, are given some kinetic energy by an accelerating voltage (U) the velocities of the ions will be different, based on their masses. Ions are separated over a fixed flight path distance according to the mass to charge ratios.

Table 1.2. Typical conditions for SF-ICP-MS

Parameter	Setting	
ICP radio frequency (RF) power	1250	W
Cool gas flow rate	15.50	L min^{-1}
Auxiliary gas flow rate	0.80	L min^{-1}
Nebuliser gas flow rate	1.00	L min^{-1}
Sample cone	Ni or Pt with B	
Skimmer cone	Ni or Pt 'H' with B	
Detection mode	Triple	
Resolution		
Low	^{7}Li, ^{11}B, ^{23}Na, ^{28}Si, ^{31}P, ^{32}S, ^{35}Cl, ^{44}Ca, ^{51}V, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{85}Rb, ^{88}Sr, ^{89}Y, ^{90}Zr, ^{93}Nb, ^{95}Mo, ^{105}Pd, ^{107}Ag, ^{111}Cd, ^{115}In, ^{118}Sn, ^{127}I, ^{137}Ba, ^{182}W, ^{202}Hg, ^{208}Pb	
Medium	^{19}F, ^{24}Mg, ^{27}Al, ^{28}Si, ^{31}P, ^{32}S, ^{35}Cl, ^{44}Ca, ^{47}Ti, ^{51}V, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{69}Ga	
High	^{39}K, ^{75}As, ^{77}Se, ^{78}Se, ^{79}Br	

The double focussing principle consists of two analysers, a traditional electromagnetic and an electrostatic analyser (ESA), positioned in either the forward (ESA before the electromagnet) or reverse (ESA after the electromagnet) Nier-Johnson geometry. Because the energy dispersion of the electromagnet and ESA are equal in magnitude but opposite in direction, they will focus both the ion angles (first focus) and ion energies (second focus). After ions are sampled in the plasma and accelerated in the ion optics region to a few kilovolts, they are focussed through an entrance slit after which they enter the mass analyser. For an instrument set up with a reverse Nier-Johnson geometry, ions enter a magnetic field which is dispersive with respect to momentum (and therefore ion mass) and focuses all ions with diverging angles of motions from the entrance slit. Ions exit the magnet and enter the ESA, which is dispersive only with respect to ion energy, after which ions are focussed onto an exit slit where the detector is positioned. Ions deflect off a plate into a conventional dynode, releasing electrons from its surface following collision. These secondary electrons released by the conventional dynode then collide with a secondary electron multiplier (SEM), composed of 19 dynodes. When electrons strike each dynode of the SEM, an electrical pulse is generated and counted with a digital counter, and multiple secondary electrons are released into the subsequent dynode until they arrive to the output electrode, which produces a signal that is transferred to a computer. The SEM detector can

operate in two modes of counting depending on detection count rate. As a result, the SEM can have a dynamic range of 10^9 allowing for quantification of both trace and major elements in a single analysis. For low count rates (< ~5 million cps) counting mode is used whereas the analogue mode can be used for higher count rates (~ 50 k - ~ 5 billion cps). A greater dynamic range can also be detected with use of a Faraday cup, increasing the range of detection to 10^{12}. The ion beam enters the Faraday cup at the open end and signals are detected by a charge integration amplifier, at the closed end. The detection mode selected is dependent on the count rate and can switch between SEM (counting and analogue mode) and Faraday cup in less than 1 ms.

1.3.5 Analysis

Elemental detection of fifty six elements was performed using a Thermo Element 2XR sector-field ICP-MS instrument (Thermo Scientific, Bremen, Germany). The instrument was fitted with either Ni sampler and skimmer cones (Spectron, Venture, CA, USA) (Chapters 2, 3 and 4), or Pt (Chapter 5) sampler and skimmer cones (Spectron, Venture, CA, USA), and a sample introduction system that consisted of cyclonic spray chamber (Glass Expansion, Victoria, Australia) and Conikal 1000 µl min^{-1} nebuliser (Glass Expansion, Victoria, Australia). The gas flows were tuned for maximum signal intensity with typical values for cool, auxiliary and nebuliser gases of 15.50, 0.80 and 1.00 L min^{-1} respectively.

Typical instrument parameters can be found in Table 1.2. Instrument parameters for samples analysed in each study, that deviate from the typical values were:

Chapter 2: The instrument was fitted with Ni sampler and skimmer cones and thirty eight elements were analysed with the ICP-MS operating in low resolution mode to monitor B, Na, Rb, Sr, Y, Zr, Nb, Mo, Pd, Ag, Cd, Sn, I, Ba, W, Hg and Pb, medium resolution to monitor F, Mg, Al, Si, P, S, Cl, Ca, Ti, V, Cr, Mn, Fe, Co, Cu, Zn and Ga and high resolution to monitor K, As, Se and Br. Samples were analysed with a calibration range of 0 – 1000 $ng.mL^{-1}$. Samples were analysed in participant number order. Samples obtained from the placebo sessions were analysed first followed by stress session samples.

Chapter 3: The instrument was fitted with Pt sampler and skimmer cones.

a. Skin patch blanking: Twenty four elements were analysed with the ICP-MS operating in low resolution mode to monitor B, Na, Mo, In, Sn and I medium resolution to monitor Mg, Al, Si, P, S, Cl, Ca, V, Cr, Mn, Fe, Co, Ni, Cu and Zn high resolution to monitor ^{39}K, ^{78}Se and ^{79}Br. Samples were analysed with a calibration range of 0 – 100 $ng.mL^{-1}$.

b. Skin patches: The nebuliser gas flow was tuned to 1.00 $L.min^{-1}$ for maximum signal intensity. The ICP-MS was operated to detect twenty four elements, using low, medium and high resolution as described in a) above. Participant patch samples, blank patches and field blank patches were also analysed.

c. Saliva samples: Tuned for maximum signal intensity, the nebuliser gas flow was 0.913 $L.min^{-1}$. Detecting twenty seven elements, the instrument was operated in low resolution to monitor Li, B, Na, As, Sr, Mo, In, Sn, I and Pb, medium resolution to monitor Mg, Al, Si, P, S, Cl, Ca, V, Cr, Mn, Fe, Co, Ni, Cu and Zn, and high resolution to monitor K and Se. A calibration range of 0 – 125 $ng.mL^{-1}$ was used to analyse samples. 60 $ng.mL^{-1}$ In was added to all samples as an internal standard and all samples were analysed three times (internal replicates). Also, external replicates of available samples were analysed too.

d. Urine samples: The nebuliser gas flow was tuned to 0.832 $L.min^{-1}$ for maximum signal intensity. The ICP-MS was operated to detect twenty seven elements; in low resolution to monitor Na, Mo, Ru, Cd, In, Sn, W and Hg, in medium resolution to monitor Mg, Al, Si, P, S, Ca, V, Cr, Mn, Fe, Co, Ni, Cu and Zn, and in high resolution to monitor K, As, Se, Se and Br. A calibration range of 0 - 125 $ng.mL^{-1}$ was used to analyse samples. 50 $ng.mL^{-1}$ Ru was added to all samples as an internal standard and all samples were analysed three times (internal replicates). Also, external replicates of available samples were analysed too.

e. Plasma samples: With the nebuliser gas flow tuned to 0.965 $L.min^{-1}$ for maximum signal intensity, the ICP-MS was operated to detect fifty five elements. Low resolution was used to monitor Li, Be, B, Na, Sc, Ti, Ga, Ge, As, Rb, Sr, Y, Zr, Nb, Mo, Ru, Rh, Pd, Ag, Cd, In, Sn, Sb, Te, I, Cs, Ba, La, Hf, Ta, W, Re, Os, Ir, Pt, Au, Hg, Tl, Pb and Bi, medium resolution was used to monitor Mg, Al, Si, P, S, Cl, Ca, V, Cr, Mn, Fe, Co, Ni, Cu and Zn, and high resolution was used to monitor K, Se and Br. Three internal replicates were analysed as well as an external standard containing 5 $ng.mL^{-1}$ of MES1, MES2 and MES4 (Spex Certi Prep) which was analysed between each sample to monitor instrument stability.

Chapter 4: Elemental detection of twenty six elements was performed. The instrument was fitted with Pt sampler and skimmer cones. Nebuliser gas flows were tuned for maximum signal intensity at 0.686 $L.min^{-1}$. The instrument was operated in low resolution mode to monitor Li, B, Na, As, Sr, Mo, In, Sn, I and Pb, medium resolution to monitor Mg, Al, Si, P, S, Ca, V, Cr, Mn, Fe, Co, Ni, Cu and Zn and high resolution to monitor K and Se. Samples were analysed with a calibration range of 0 – 100 $ng.mL^{-1}$. For each sample three intra replicates were analysed to obtain greater accuracy and precision. 2% HNO_3 was analysed as the solvent blank. An external calibration sample set, to quantify sample element concentration,

was analysed every three samples. This was analysed a total of three times using a bracketing technique: as the first and last sample and also after every three participant samples of each analysis sequence (e.g. standard, sample, sample, sample, standard).

Chapter 5:

Section 5.4 Elemental detection of Mn was performed with the ICP-MS instrument operating in medium resolution. The instrument was fitted with Ni sampler and skimmer cones. The nebuliser gas flow, tuned for maximum signal intensity, was 1.131 $L.min^{-1}$. The instrument was operated in medium resolution to monitor Mn. A sample containing 10 $\mu g.mL^{-1}$ was analysed in order to conduct semi-quantitation. Three intra replicates were taken for each sample analysed.

Section 5.5 Same parameters as those used in section 5.4 above except the instrument was fitted with Pt sampler and skimmer cones. Three intra replicates were taken for each sample analysed and samples were semi-quantified by taking the ratio of intensity obtained for the known Mn concentration of 100 $\mu g.mL^{-1}$ to those obtained for each sample.

Section 5.6 The instrument was fitted with Pt sampler and skimmer cones. Elemental detection of twenty six elements was performed. The ICP-MS was operated in low resolution mode to monitor Rb, Sr, Nb, Mo, Pd, Ag, Cd, Sn, Ba and Pb, medium resolution to monitor Mg, Al, Si, P, S, Ti, V, Cr, Mn, Fe, Co, Ni, Cu and Zn, and high resolution to monitor K and Br. Samples were analysed with a calibration range of 0 – 100 $ng.mL^{-1}$. A 2% HNO_3 solvent blank was analysed using a bracketing technique that enabled analysis after every two participant samples (e.g. blank, sample, sample, blank) to check the baseline element levels of the instrument. Also, all samples were analysed in triplicate (intra replicates) to estimate instrumental precision.

Section 5.7 The instrument was fitted with Pt sampler and skimmer cones. The nebuliser gas flows were tuned for maximum signal intensity with a value 0.686 $\mu l\ min^{-1}$. Twenty three elements were analysed. The instrument was operated in low resolution mode to monitor Rb, Sr, Nb, Mo, Ag, Cd, Ba and Pb, medium resolution to monitor Mg, Al, Si, P, S, Ti, V, Cr, Mn, Fe, Co, Ni, Cu and Zn, and high resolution to monitor K. Samples were analysed with a calibration range of 0 – 100 $ng.mL^{-1}$. A 10 $\mu g.ml^{-1}$ standard was analysed after every sample to ensure the instrument remained stable throughout the analysis sequence. Also, all samples were analysed in triplicate (intra replicates) in order to estimate instrumental precision. Field blanks, EDTA sampling solvent blank, a 2% HNO_3 blank and a DI H_2O blank were also analysed.

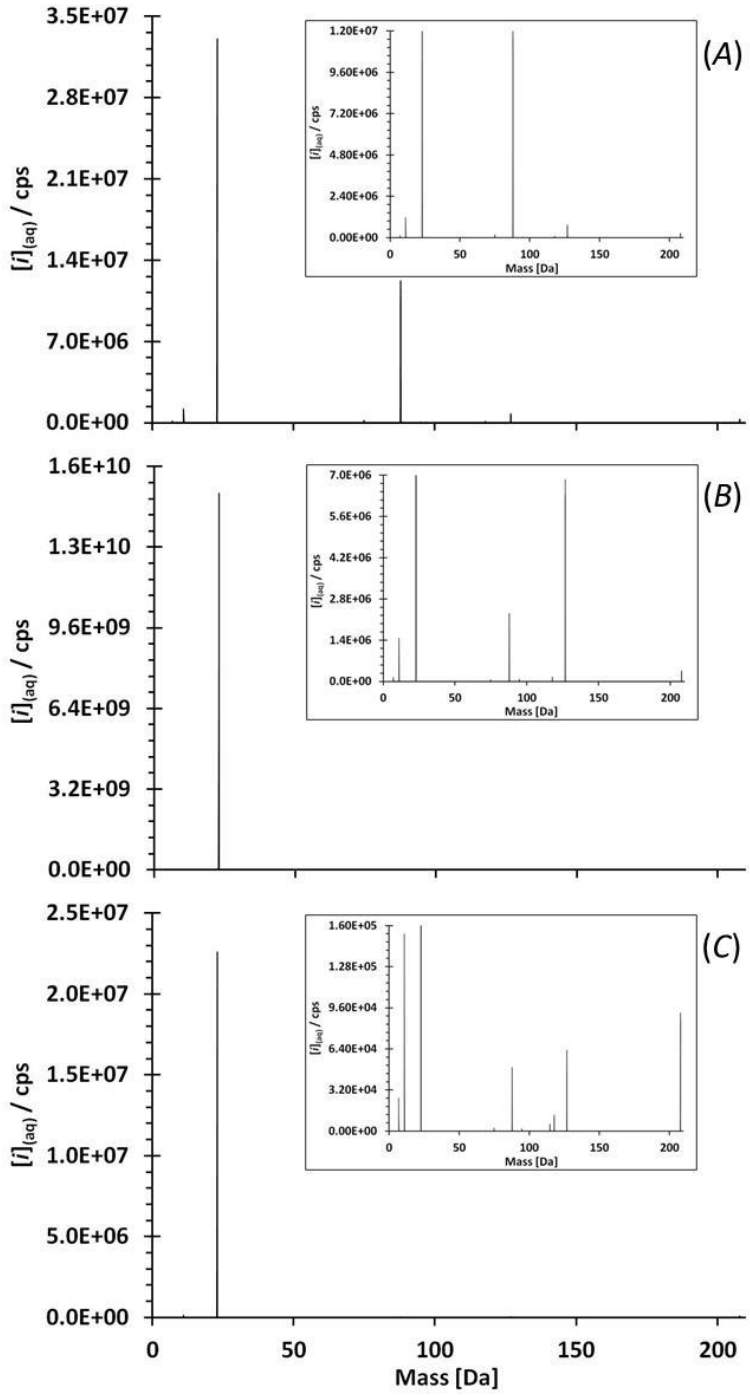


Figure 1.4. An example of saliva profiles from emotional stress, from the study in Chapter 4, showing intensity of elemental masses from 0-210 Da for (A) Post intervention B sample (B) Pre intervention B sample and (C) Blank B sample. Inserts show magnified range with second highest intensity as max

Sample analysis order was randomised to remove systematic errors. Unless stated otherwise, samples analysed by ICP-MS were randomised in order to avoid bias. Sample identification numbers were listed in Microsoft Excel 2010 and were assigned a random number using the random number function in the software (=RAND()). These random

numbers were sorted from lowest to highest using the sort function to determine the order samples were to be analysed.

Using the instrument software live data and mass abundance was viewed (Figure 1.4). The μ, σ and RSD of the sample intensities were recorded automatically for each of the selected elements onto Microsoft Excel spread sheets along with the sample ID numbers.

1.3.6 Data processing

Signal intensity (counts per second) was obtained as ICP-MS raw data. Internal replicate averages (n=3) were initially calculated. Concentrations, limits of detection and quantification of the sample elements were then determined using linearity curves, y = mx + c (where y = intensity, m = gradient, c = intercept and x = unknown), obtained from constructing intensity against concentration for each element present in the calibration standard (Figure 1.5). Sample dilutions were then factored into the final sample concentration. Internal standards were also used to semi-quantitate concentration by calculating the known amount of internal standard added and using the ratio between this known value and that observed. Element concentrations obtained for the blank sample were then subtracted from all sample element concentrations. All element concentrations in samples where either blank sample elemental concentrations were higher than those observed or the limit of quantification were higher, were assigned a concentration 0 $ng.mL^{-1}$.

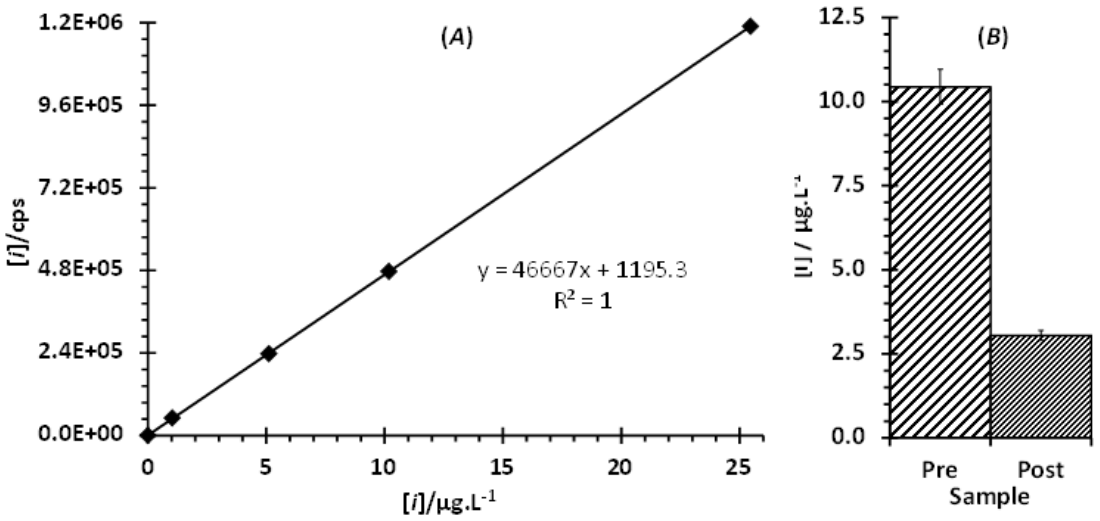


Figure 1.5. (A) An example of a calibration graph using intensity acquired (cps) against standards with known concentration ($ng.mL^{-1}$). (B) An example of the average calculated concentration of pre and post samples, with standard error of the mean (95% confidence), in pre and post intervention saliva collected, from the emotional stress study in Chapter 4. This data refers to the element Mn.

1.4 Electrospray Ionisation Mass Spectrometry

Electrospray ionisation (ESI) is a widely used technique for protein analysis for samples in liquid form that are stable, not in an excited state, can be unlimited in mass and have a high ionisation efficiency. ESI emerged as a powerful method for producing intact ions in vacuo from large complex species in solution following an earlier approach to ionisation by Dole.[66, 67] Fenn *et al*. showed that multiply charged ions were obtained from proteins, allowing molecular weight determination with instruments with limited mass ranges to as low as 2000 m/z. Fenn overcame previous issues with the analysis of volatile and semi volatile compounds e.g. small, low molecular weight compounds, that could be readily transferred to the gas phase without breaking.

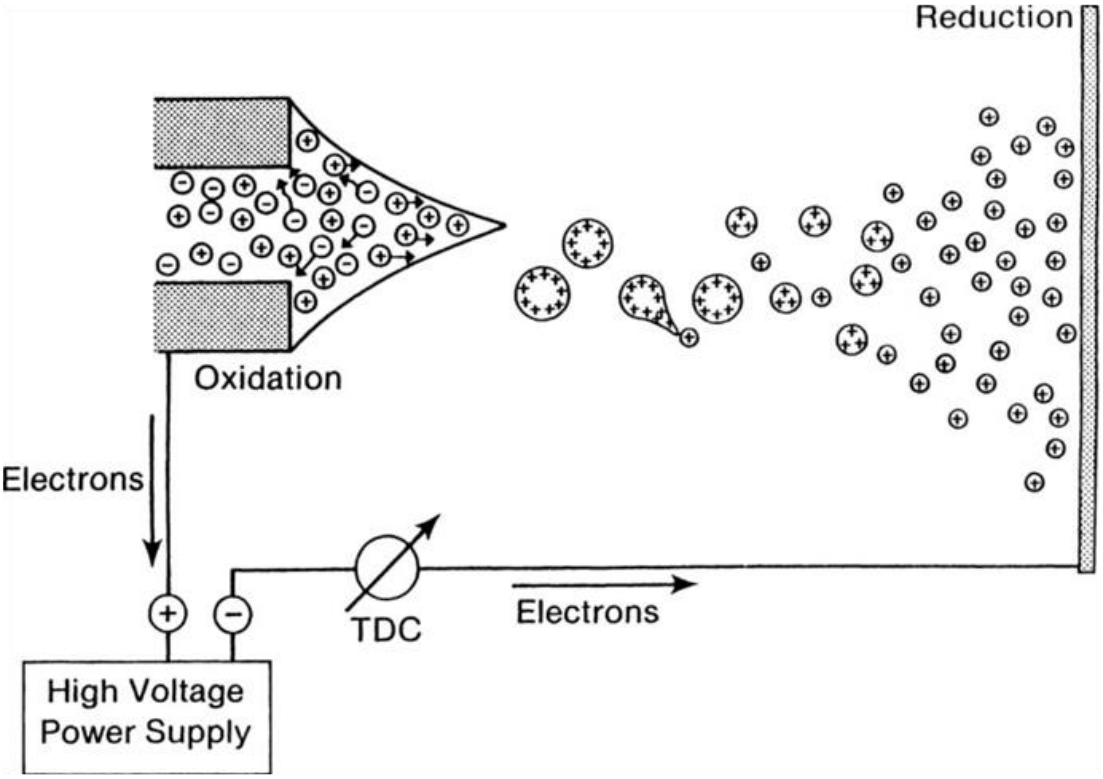


Figure 1.6. Formation of electric double layer in liquid produced as a result of the penetration of the imposed electric field into liquid[68]

There are two major ionization mechanisms thought to be associated with ESI-MS, the ion evaporation and charged residue models. The ion evaporation model implies that ions are expelled due to Coulombic forces. As the droplet decreases and reaches a certain radius the field strength at the surface of the droplet becomes large enough to assist the field in desorption of solvated ions. The charge residue model suggests that as the droplets shrink the field strength at the location with the highest curvature forms a Taylor cone.

Consequently, highly charge smaller droplets are emitted from the Taylor cone. This is repeated until only one analyte molecule is present in droplets.

A strong electric field, under atmospheric pressure, is applied to a liquid passing through a capillary tube with a weak flow (1-10 µl min^{-1}). The electric field is obtained by the application of a potential difference of 3-6 kV between the capillary and a counter electrode (0.3-2 cm apart) resulting in electric fields of the order 10^6 V m^{-1} (Figure 1.6). As a result of the field, a charge is accumulated at the liquid surface located at the end of the capillary, forming a Taylor cone which eventually breaks to form highly charged droplets (Figure 1.7). A volatile charge modifier is added to the sample solution producing ionised analyte species. Common modifiers used are ammonium for negative ions or formic acid for positive ions. A coaxial gas is injected at a low flow rate allowing the dispersion of the spray to be limited in space. The droplets then pass through a heated capillary or a gas curtain, usually N_2, to remove remaining solvent molecules.[69]

The 'Rayleigh limit' is when an equilibrium is formed between surface tension forces and the ion repulsion within the droplet i.e. as the solvent evaporates, the drop shrinks and becomes unstable. Gomez and Tang concluded the breakdown of droplets can occur before Rayleigh limit is reached because the droplets are mechanically deformed. Consequently there is a reduction in the repulsion necessary to break down the droplets.[70]

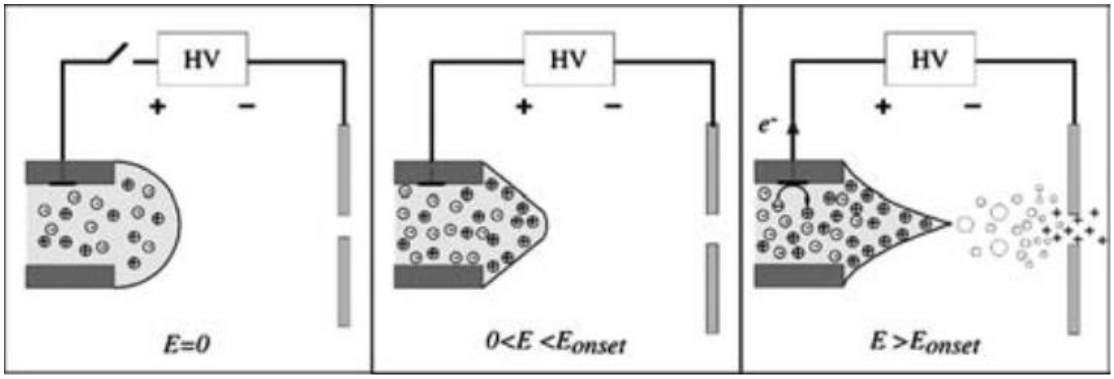


Figure 1.7. Formation of electrospray droplets resulting from increasing electric fields[71]

Although ESI allows for the analysis of non-volatile species and small molecules, a disadvantage is the result of ion suppression as a result of competition for charge. However, pre-fractionation using sample purifying techniques, such as high performance liquid chromatography (HPLC), can be used.[72]

1.4.1 Analysis

Samples in Chapter 5 sections 5.8 were analysed by direct injection electrospray mass spectrometry (ESI-MS) on a Waters Synapt G1 HDMS Time of Flight (TOF) mass spectrometer in firstly negative and then positive ion modes. Samples were analysed for two minutes over a scan range of 50 m/z to 4000 m/z for negative mode and 40 m/z to 4000 m/z in positive ion mode. The resolution of the instrument in V mode is between 8,000 and 10,000. Other important parameters can be observed in Table 1.3.

All samples were analysed with the enhanced duty cycle (EDC) on. The EDC sets the instrument quadrupole over a specified region and thus enhances detection over a small window, of about m/z 70. Before a collected sample from the study was analysed, the lines were cleaned using the same solvent of EtOH that was used for skin sample collection. This was accomplished by drawing 1 ml of the solvent into a syringe that was then placed on a pump. Prior to placing the syringe onto the pump, approximately 0.01 ml of solvent was manually pushed through the sampling capillary to remove memory effects from previous samples. As a result, a low background could be maintained.

Table 1.3. ESI-MS experimental parameters for the profiling of the dorsal (TF) and plantar (BF)

Parameter	Level		Units
Polarity	**ES-**	**ES+**	
Analyser	V mode	V mode	
Capillary	3.0	3.0	kV
Sampling cone	40.0	30.0	V
Extraction cone	4.0	2.5	V
Source temperature	**120**	**120**	**°C**
Desolvation temperature	**300**	**300**	**°C**
Cone gas flow	0.0	0.0	$L.hr^{-1}$
Desolvation gas flow	600	500	$L.hr^{-1}$
Sample flow	5.0	5.0	$\mu l.min^{-1}$
Start mass	50	40	Da
End mass	4000	4000	Da

Once the capillaries had been purged with EtOH the syringe was purged with the next sample to be analysed. This was achieved by drawing up the sample and injecting it to waste three times. The sample was then loaded into the syringe and placed onto the pump as previously carried out. When a stable signal was observed in the instrument software, data collection commenced and at least 60 scans (Figure 1.8) were collected over a two minute analysis time.

As well as analysing EtOH solvent (sample blank) in between each sample analysis, a pre-made calibration solvent of 0.5M sodium formate was analysed every ten samples ensuring that drift of the observed m/z of ions was kept to a minimum, obtaining accurate data as a result.

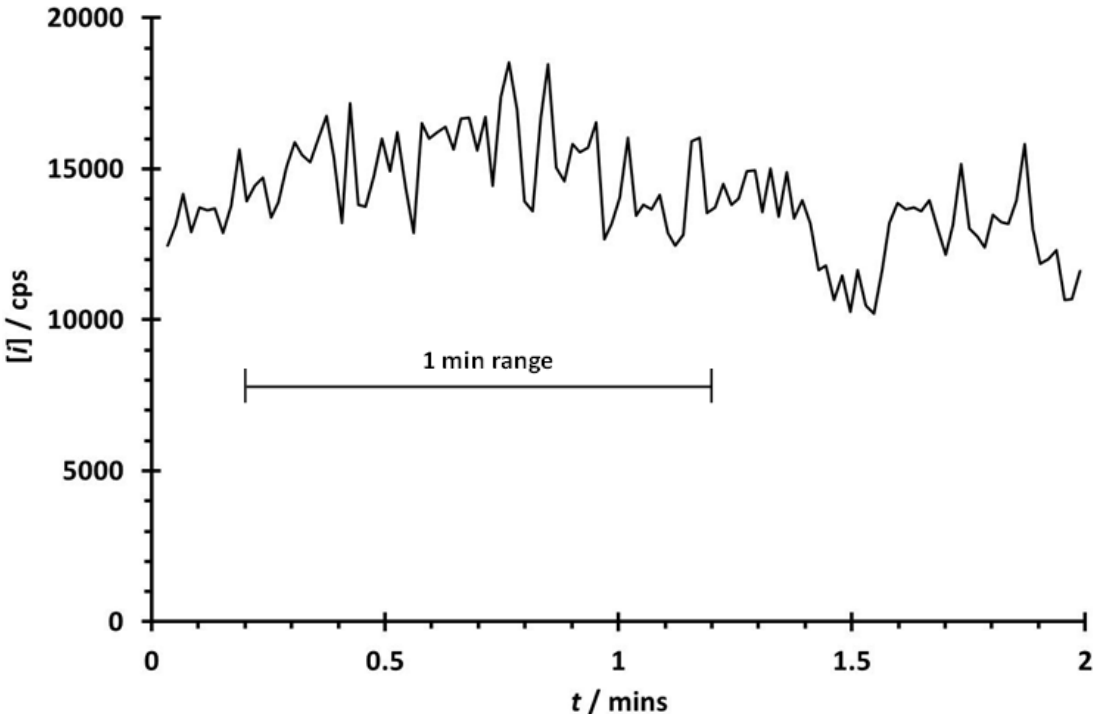


Figure 1.8. An example of the total ion current (TIC) obtained from ESI-MS negative ion mode for participant UF3, from the study in Chapter 5 section 5.8, plantar (BF) sample range 0.0-2.0 mins and range selected 0.2-1.2 mins

A randomised order to analyse the samples was determined in order to avoid bias, using the procedure described in section 1.3.5.

1.4.2 Data Processing

Sample data was initially prepared using Waters MassLynx V4.1 (Waters). Opening the raw data file in the software produced a chromatogram. A stable range of one minute was averaged from the chromatogram (Figure 1.8) producing a spectrum showing the peak intensity (area) against the observed m/z. From this spectrum, a lock mass correction with mass m/z 112.9851 and settable parameters of Np 0.700 and resolution 8000 were selected. Consequently, a second spectrum was produced showing the intensity (centroid height) against the observed m/z (Figure 1.9).

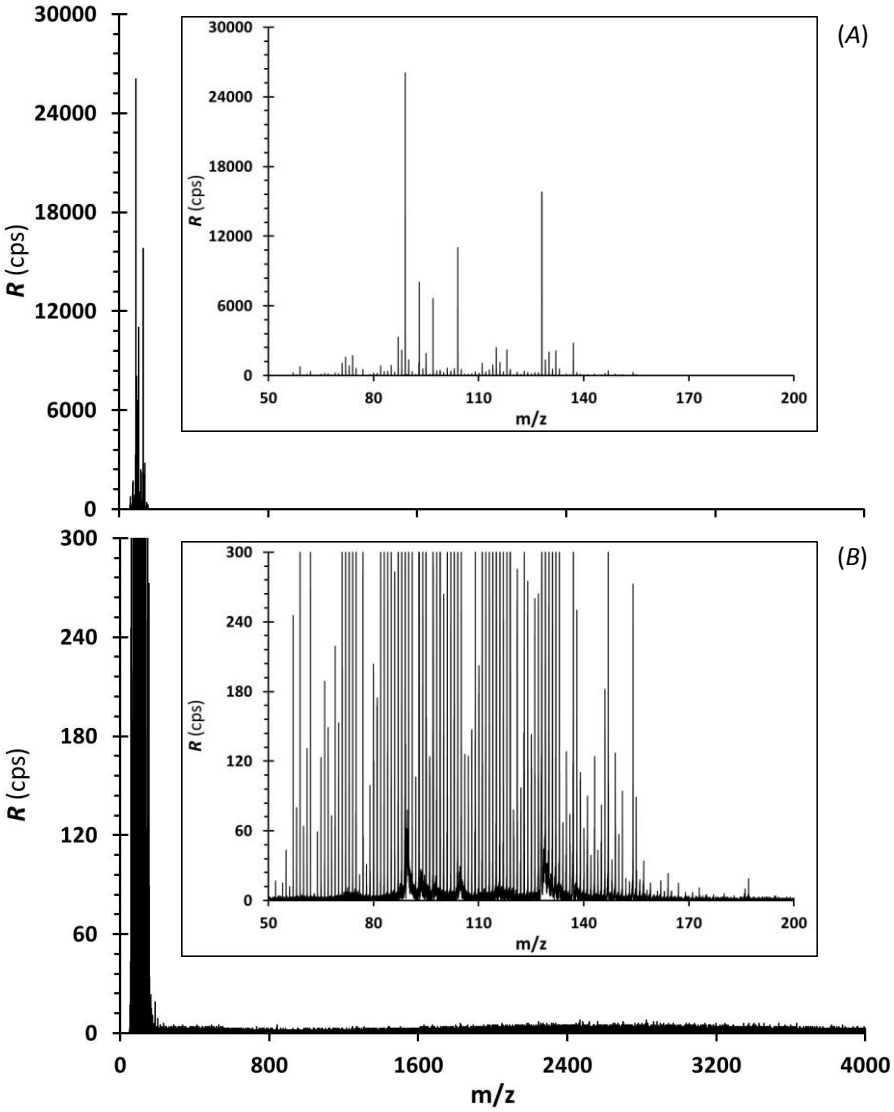


Figure 1.9. An example of a negative ion mode mass spectra, m/z 0-4000, of VOC in skin profiles obtained from the plantar (BF) site of participant UF3, from the study in Chapter 5 section 5.8, using the LSP. (A) Profile (B) 100 x magnified profile, all inserts m/z 50-200

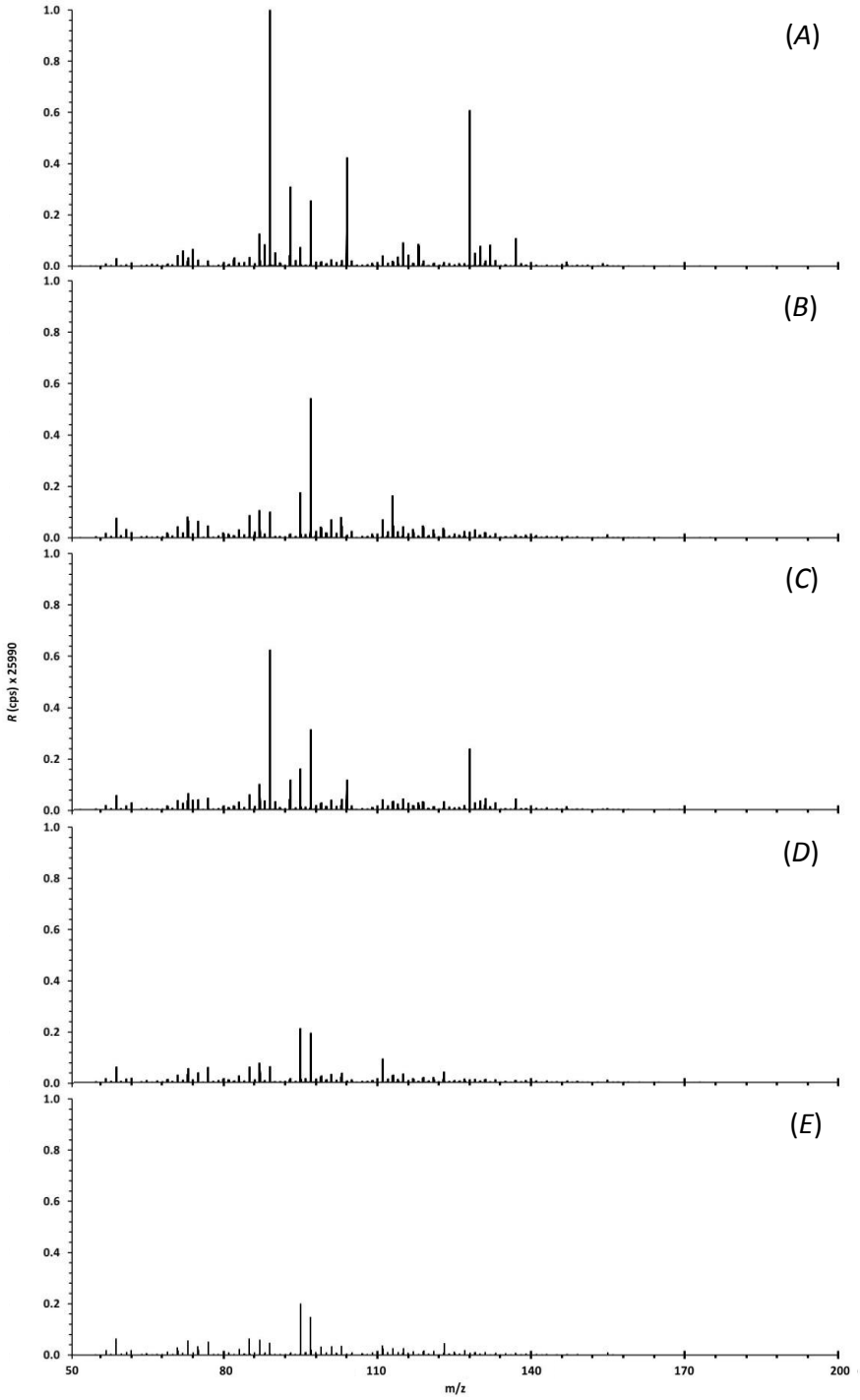


Figure 1.10. An example of spectra for participant UF3, from the study in Chapter 5 section 5.8, m/z 50-200: A = Plantar (BF), B = Plantar (BF) blank, C = Dorsal (TF), D = Dorsal (TF) blank, E = Blank. Intensity normalised to 1 from 25990 counts

Using the example the study in Chapter 5 section 5.8, five spectra for each sample obtained from one participant were observed together (Figure 1.10) and were then overlaid (Figure 1.11) to gain a visual representation of the differences. This was performed for samples from each participant.

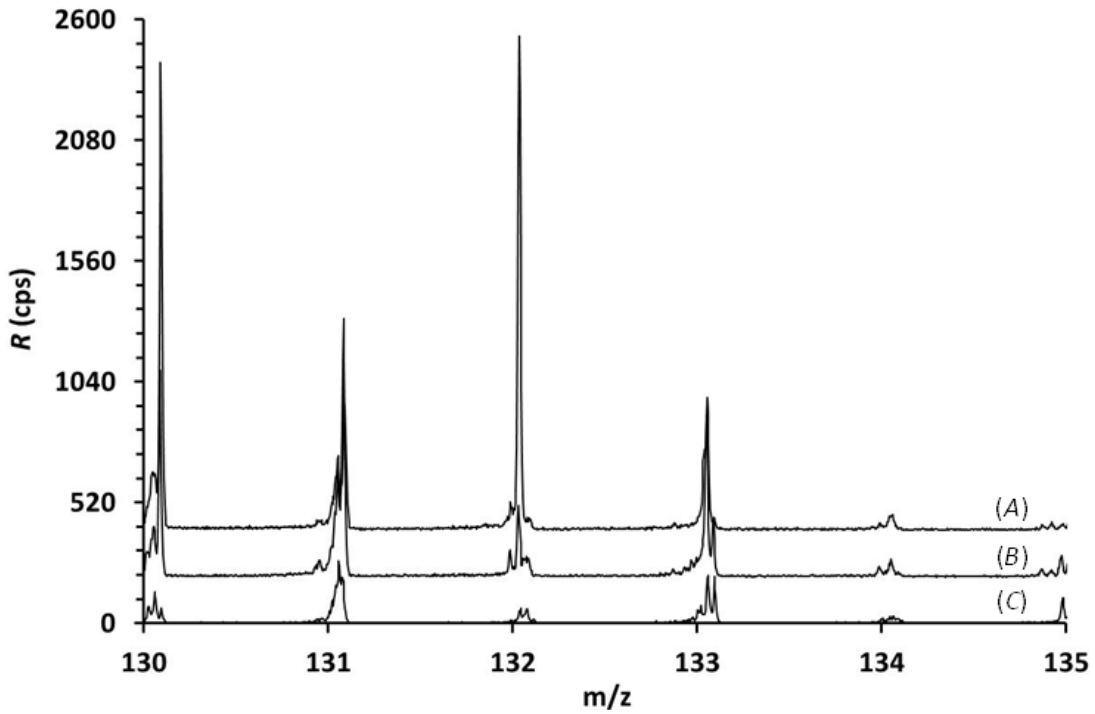


Figure 1.11. An example of mass spectra m/z 130-135 from participant UF3, from the study in Chapter 5 section 5.8, (A) Plantar (BF) sample offset by 400 cps (B) Dorsal (TF) sample offset by 200 cps (C) Blank sample

Ion intensities obtained from the solvent sample (EtOH) were then subtracted from all fifty sample values and any negative values observed were substituted by "<LOD". Following this, the LSP blank sample intensities were then subtracted from their associated participant samples and all negative values were substituted by "<LOD". Using participant 1 as an example, the intensities obtained for the dorsal LSP blank (UF1 TF PB) were subtracted from the intensities obtained for the dorsal (UF1 TF) sample and the intensities obtained for the plantar (BF) LSP blank (UF1 BF PB) were subtracted from the intensities obtained for the plantar (BF) (UF1 BF) sample for each observed ion. This was performed for each set of data obtained from each participant. Finally, intensities obtained for the field blank (FB) were subtracted from their associated sample set and all negative values were substituted by "<LOD". Again using participant 1 as an example, the intensities for the field blank (UF1 FB) were subtracted from all other samples obtained for participant 1 (UF1 TF PB, UF1 TF, UF1 BF PB and UF1 BF).

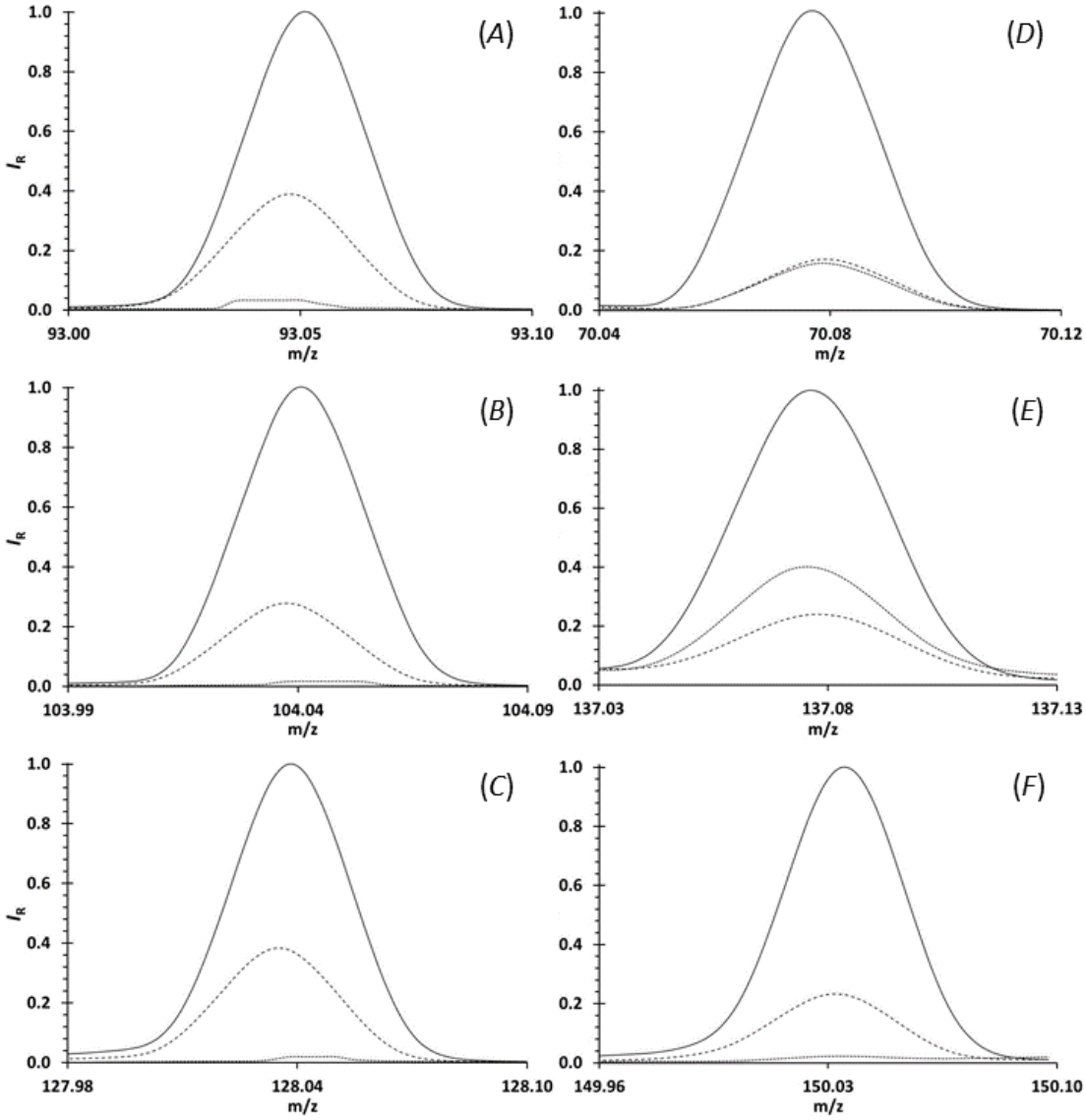


Figure 1.12. An example of overlaid extracted ion chromatograms showing responses for plantar (BF) (solid line), dorsal (TF) (dashed line) and blank (dotted line) sample sites in ESI-MS negative ion mode: (A) m/z 93.0346 (B) m/z 104.0354 (C) m/z 128.0354 and ESI-MS positive ion mode: (D) m/z 70.0783 (E) m/z 137.0760 (F) m/z 150.0297 from participant UF3 (Male), from the study in Chapter 5 section 5.8. The intensities displayed (I_R) have been normalised with respect to the plantar (BF) sample site peaks

1.5 Nano-Electrospray Ionisation Mass Spectrometry

Mass spectra is obtained from this electrostatic ion trap using Fourier Transform (FT) based on a new concept by Marakov.[73–75] The trap consists of an external barrel shaped electrode that is cut into two equal parts with a small interval and a spindle shaped central electrode (Figure 1.13). The maximum diameters of the outer and inner electrodes are 20 mm and 8 mm, respectively. Ions are injected tangentially, with some kiloelectronvolts through the interstice between the two parts of the external electrode which is at ground potential. Several kilovolts (electrostatic voltage) are applied to the central electrode, negative for

positive ions. Following injection, ions start to oscillate in intricate spirals around the central electrode along the *z axis* under the influence of an electrostatic field.

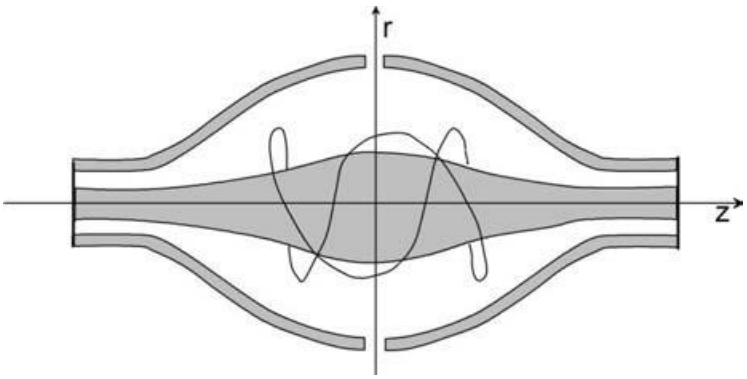


Figure 1.13. The electrostatic trap ('orbitrap') according to the description of Makarov.[74] An outer electrode has the shape of a barrel cut into two parts separated by a small gap. Inside is a second spindle shaped electrode. Only DC voltages are applied. Ions turn around the central electrode while oscillating back and forth along the *z axis* resulting in a movement of intricate spirals.[69]

The frequency of this oscillation is directly linked to the mass to charge ratio and is independent of the kinetic energy of the injected ions. The broadband current of these oscillating ions is then measured and converted to FT to individual frequencies and intensities, generating a mass spectrum.

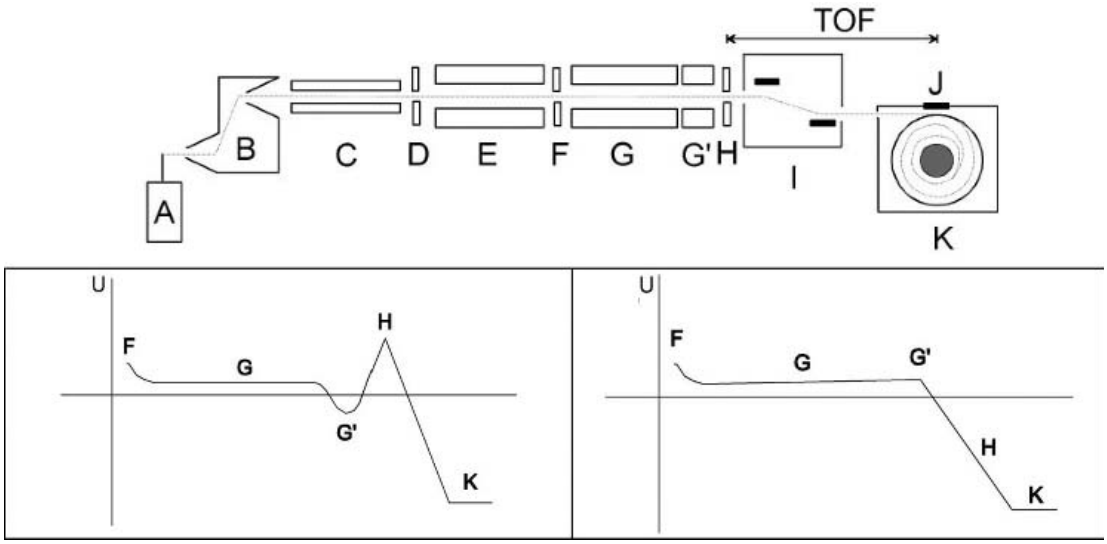


Figure 1.14. A complete stack (includes atmospheric pressure source, analytical quadrupole, a storage linear trap and a quadrupole itself) of the orbitrap coupled to an electrospray source at (A) atmospheric pressure *z* spray (2 mbar). C: Guide ion (RF quadrupole, 10^{-2} mbar). D: Lens. E: transport RF quadrupole (10^{-5} mbar). G and G': segmented trapping quadrupole to store ions to inject (10^{-5} mbar). F and H: lenses. I: Ion beam deflection device prior to injection into orbitrap. J: Repeller electrode assists ions enter the trap and spiral down the field to orbit around the spindle shaped electrode. K: Side view cut of the orbitrap cut at the level of the gap between two half barrel electrodes.[69]

Ions are produced and focussed from A to F by classical atmospheric pressure source coupling (Figure 1.14). Ions are allowed to accumulate close to the exit of the orbitrap in the

ion trap by adjusting the potentials on the F electrode and on the last segment G' of the trap; allowing rapid injection. From H to J, ions fly freely except for a beam deflection, to avoid helium injection, from the linear trap in the orbitrap. This field free region has a focus plane twice the length of the acceleration region, where ions of the same mass are focussed successively. Distance H to J is adjusted to correspond this distance allowing the injection of ions of one m/z at a time, focussed in a short period of time of nano seconds resulting in a total injection time of about 1 µs for successively higher m/z ions. Consequently, ions of the same mass group efficiently while dispersing ions of different masses to reduce space charge effects, resulting in improved detection. Due to the injection time of the ions with the same m/z being shorter than the time of one oscillation along *z*, movement of ions are very coherent resulting in very sensitive detection. This coherence is not observed around the central electrode therefore a reduced background is obtained. The good coherence also improves mass resolution and accuracy.

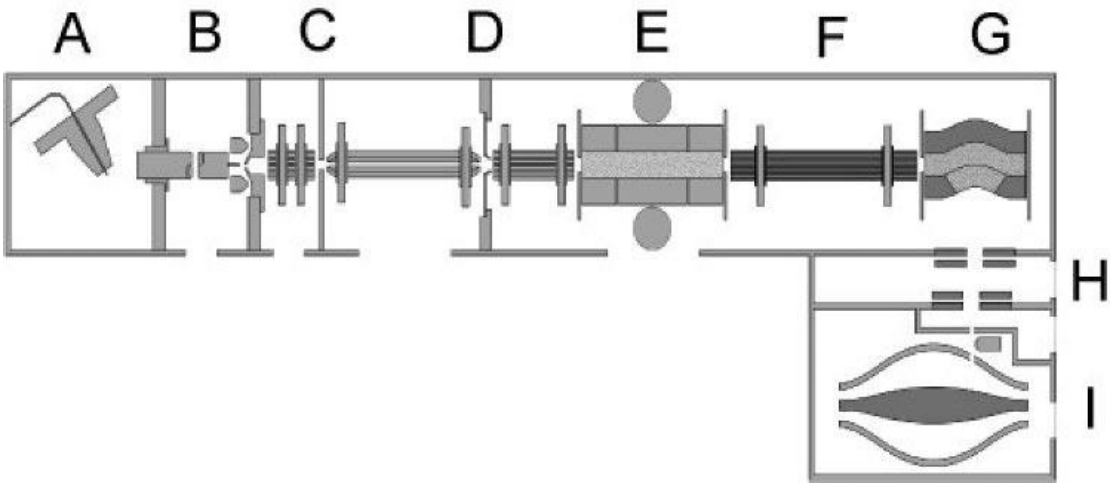


Figure 1.15. Schematic of Thermo Electron Corporations Orbitrap. A: Atmospheric pressure source. B: Heated capillary. C: Several multipole focussing devices. D: Gating lenses. E: Focussing octapole. E: Ion trap with two detectors. F: Focussing multipole. G: 'C-trap' quadrupole.[69]

Commercially available orbitrap instruments, by Thermo Electron Corporation (June 2005), uses a linear ion trap (LIT) that is used for ion storage and injection to the orbitrap by axial ejection, independently used as a linear trap by radial ejection (Figure 1.15). It is possible to inject all the ions from a LIT, or selective ones. A normal acquisition cycle time in the orbitrap is 1 second. Other operations can be performed in the orbitrap during this cycle time, after ejection to the orbitrap. For instance obtaining low resolution spectra from the LIT while acquiring a high resolution spectrum from the orbitrap. The gating lens before the LIT allows regulation of the number of ions injected and this allows optimal ions injected in the orbitrap resulting in optimised performance and also avoiding space charge effects.

1.5.1 Analysis

From Chapter 5 section 5.8, accurate mass determination of four plantar (BF) samples from randomly selected participants (UF1, UF4, UF5 and UF8) were analysed in an attempt to determine the mass accuracy, as well as the identity, of ions by direct injection nano-electrospray mass spectrometry (nESI-MS) using a LTQ orbitrap mass spectrometer (Thermo Fisher Scientific, UK) in both negative mode and positive ion modes with the resolution of the instrument between 50,000 to 100,000 (Table 1.4).

Table 1.4. nESI-MS experimental parameters for the profiling of the dorsal (TF) and plantar (BF), from the study in Chapter 5 section 5.8

Parameter	Level		Units
Polarity	ES-	ES+	
Scan Low Mass	50	50	Da
Scan High Mass	1000	300	Da
Analyser Temperature	28.64	26.7	°C
Capillary Voltage	92.5	-77.5	V
Spray Voltage	1487.8	1465	V
Spray Current	0	0	µA
Capillary Temperature	190.1	190.0	°C
Sweep gas flow rate	0.07	0.13	

Data obtained from nESI-MS was evaluated in the instrument software allowing the discovery of possible formulae, magnitude of error as well as the accurate mass for the ions of interest.

1.6 Statistical Methods

Data obtained from sample analysis by the mass spectrometry techniques, described in this research, was collected, organised, analysed, interpreted and modelled using statistical packages such as Umetrics Simca-P+ 12.0.1., Microsoft Excel 2010 or instrument software. These packages provided the tools to examine all data samples in a population that assisted in describing the nature of the data analysed, explored the relation of the data to the underlying population, created models to present how the data related to the underlying presentation, proved (or disproved) the validity of the data and predicted analytics to anticipate future predictions.

1.6.1 Multivariate Analysis

The principle of multivariate analysis (MVA) is the simultaneous, statistical study of a number of variables with the aim to extract information out of existing multivariate data. MVA essentially models reality where each situation involves more than a single variable.

Today's information age has resulted in masses of data in every field that are multivariate due to the availability of instrumentation and complexity of systems and processes. Continuing the old fashioned approach of uni- and bivariate analysis is often misleading and inefficient. For example, it is difficult to describe the whole picture of the process when only performing one function on the data as a lot of potentially significant results can be overlooked and also performing functions on many variables is often impractical i.e. performing t-tests on 300 variables. When there are only two variables (univariate analysis) to measure this, information can be represented graphically where coordinates of a given point give the values taken from two variables i.e. assumes X independent variables makes regression of Y observations.[76] However, graphical representations for three variables is more difficult and not possible for four or more, and it is here where computer analysis is useful in finding relationships between variables. MVA extracts information from data with multiple variables by using all the variables simultaneously. By observing all the data collectively, MVA generates models that can predict fundamental trends to identify latent variables without the loss of information.

Each sample is known as an observation whereas each analyte is called a variable. In mass spectrometry a number of variables can be produced when analysing biological samples such as skin, urine, plasma and saliva.

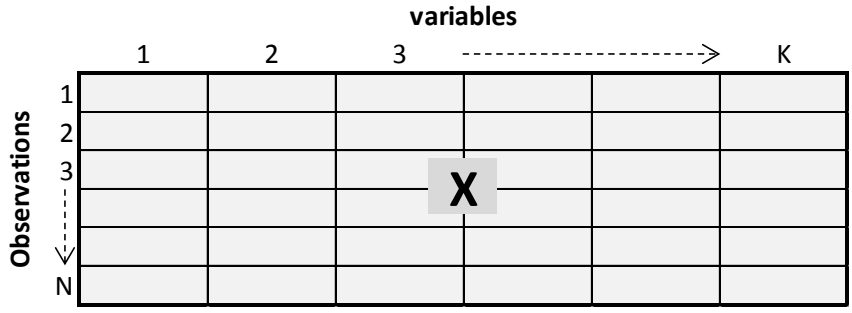


Figure 1.16. Multivariate analysis matrix template. Each row represents a sample (observation) and each column represents the corresponding variables (e.g. Element)

The major benefit of employing MVA is its ability to reduce the dimensionality of large data sets. In principal components analysis (PCA) linear independent combinations of the original variables (X_1, X_2, X_3,….X_n) are reformed into a new set of variables called principal components (PC_1, PC_2, PC_3,….PC_n), maintaining vital information from the original data set. For example, PC1 = $a_{11}X_1$ + $a_{12}X_2$ +……. + $a_{1n}X_n$. Newly created vector coefficients (a_{mn}) interpret maximum variability in the data and are independent principal components therefore are not correlated to one another. The first principal component interprets the maximum variance of the data, the second principal component interprets the subsequent

variance, the third principal components interprets the next variance, and this continues until no significant variance can be found.

Partial least-squares discriminant analysis (PLS-DA) uses a training set of data to assign a group of unknowns so that it fits the data. In order to maximise separation approximately 70% of a known variable, enabling the highest separation, is chosen following initial interpretation of known classes within the information. A model is then built and the process is iterated until cross validation is successful. Linear combinations of the original variables, X_1, X_2, X_3,….,X_n, are used to find the linear discriminant function, Y, by selecting coefficients a_1, a_2,a_3,….a_n, producing the maximum difference between groups of observations. In other words, observations in the same group will have similar Y values whereas those in different groups will have a different value for Y.[77, 78]

The supervised model, PLS-DA, and unsupervised model, PCA, are commonly employed for MVA. Use of the supervised model, where the model is created using information already known about groups that need to be discriminated, is often criticised. PLS-DA is generally utilised when no conclusion can be obtained from the initial use of PCA.

Multivariate analysis and descriptive statistics were conducted on large sets of data obtained from human samples (Chapter 2, Chapter 3 and Chapter 5 sections, 5.6, 5.7 and 5.8). The data processing procedures are outlined below using data from samples obtained from the study in section 5.8. Depending on the study and type of data, collected ions can be interchanged for elements whereas sample type (dorsal (TF) or plantar (BF)) can be interchanged with other sample types or genders.

In Chapter 5 section 5.8, MVA was performed using Simca-P+12.0.1 (Umetrics), on the blank subtracted data set to identify ions that could possibly show differences between the plantar (BF) and dorsal (TF) sample sites. All values of "<LOD" were replaced with the value 1 as the software was unable to accurately process data sets that contained empty cells or the value zero. This was due to the inability of the software's algorithm to process no value cells leading to errors. The value 1 was chosen as this low number would not prove to be significant when creating data models.

Initially, data was transformed (LOG_{10}), to normalised data, in the software and then grouped into two discriminant classes (PLS-DA), class 1 for dorsal (TF) samples and class 2 for plantar (BF) samples, with Pareto scaling (ParN) used. This type of scaling is generally used when only medium or small features in the data are important. Due to the small changes in large data sets, this method was employed. The number of significant components (A) was

calculated for the model and the resulting S-plot was used to determine the ions that would later be further inspected.

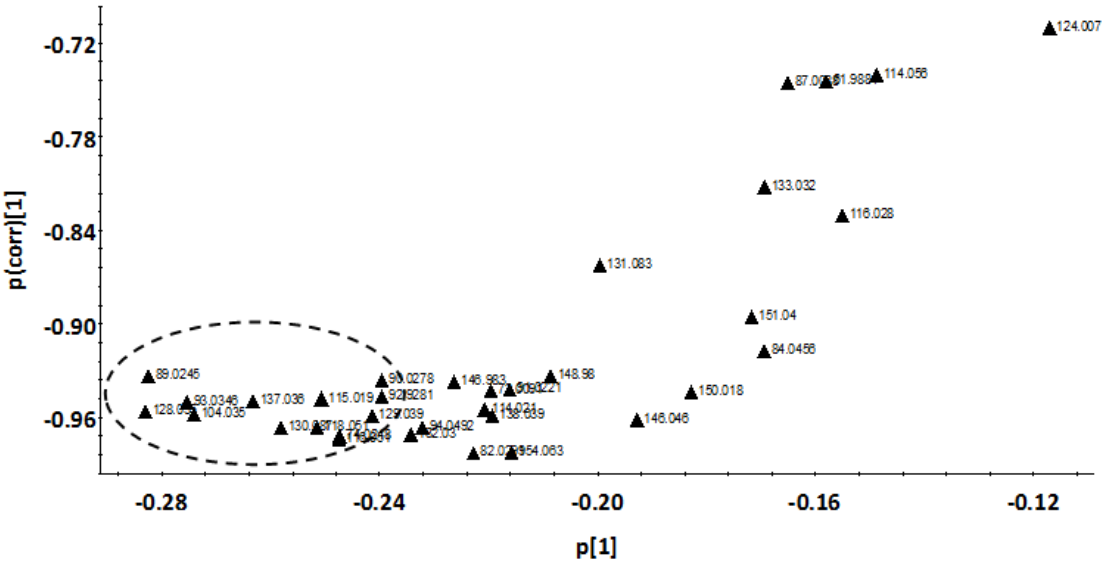


Figure 1.17. An example of a supervised model (PLS-DA) Simca-P+ S-plot (PLS-DA) obtained from 20 samples (observations) analysed by ESI-MS in negative ion mode, from the study in Chapter 5 section 5.8, R2X = 96.8%, Q2X = 89%

All ions chosen from the S-plot (Figure 1.17) were included to create a new, unsupervised model (PCA). The software calculated the number of significant components for the PCA data set and score scatter and score loading plots were constructed. From the score loading plot, the contribution of each ion towards the model can be viewed. The heavier the weight of these ions (away from the centre 0) the more likelihood distinction of observations in later modelled scatter plots using these ions. Initially, the heaviest weighted ions were chosen, as shown by the dotted ellipse in Figure 1.17, to produce further models in order to discover important less weighted variables. Using the score loading plot, the biggest weighted contributors would be removed to examine if the lower weighted ion contributors could produce clusters between groups of observations on the score loading plot. This process was iterated until no separation was observed between the two sample groups. Those ions that produced these clusters were chosen as the vital biomarkers.

1.6.2 Descriptive Statistics

Analytical methods should be free from systematic error meaning the value given for the amount of analyte should be the true value. However, even if no systematic errors are present, random errors make it unlikely that the achieved value would exactly equal to a reference amount (a standard or experimental value). In order to determine whether these differences can be attributed to random variations, significance tests can be employed.

Significance tests are testing the truth of a hypothesis which is known as the null hypothesis (H_0). The term null implied there is no difference between the observed and known (or second experimental) values other than that which can be attributed to random variation. Assuming H_0 is true, statistical theory can be utilized to calculate the probability that the differences between the observed and known values are due to random errors. The lower the probability that the differences occur by chance the less likely H_0 is true.

Tests for comparison of experimental means (t-tests), comparison of standard deviation (F-test) and analysis of variance (ANOVA) are described in the supplementary information (Chapter 1 Statistical Analysis).

1.7 Ethics

Studies were conducted in accordance with the ethical principles of good clinical practice and the Declaration of Helsinki. The research followed the protocols that were reviewed and granted favourable opinion by the local ethics advisory committee, Loughborough University; G7-P19 Chapter 2, R10-P126 Chapter 3, R11-P107 Chapter 4 and R11-P33 Chapter 5. All participants taking part in each study gave written informed consent.

Prior to sampling, all participants were provided with an information sheet outlining reasons for the study and the procedures involved and were asked to complete a health screen questionnaire upon arrival on the morning of sample collection. Participants were informed both verbally and in writing of their right to withdraw at any time during the process. Health screen questionnaires were anonymised by allocation to each participant of a Participant Number, which was used on the questionnaire sheets instead of participant's name. Any health issues were checked before proceeding with sampling. The final document presented to the participants before sample collection could proceed was the consent form. Participants filled out the important information, such as contact number in the event the participant needed to be contacted again and also the participants' signature to verify they understood the purpose of the study and agreed to participate.

At the end of the study all health screen questionnaires and consent forms were grouped and placed in a sealed envelope. Envelopes were locked in a filing cabinet and participants were told their personal information would remain confidential.

1.7.1 Recruitment of participants

For all studies participants were recruited from social networks in addition to Loughborough University staff and students. Participants sampled in Chapter 5.7 were recruited by internal

advertising within Unilever PLC, Bebington. For studies in Chapters 2, 4, 5.6, 5.7 and 5.8 all participant samples were taken in a small internal room at the Centre for Analytical Science within the Department of Chemistry at Loughborough University. For Chapter 3 all participant samples were taken at 25°C in the climate chamber in the School of Sport, Exercise and Health Sciences building at Loughborough University. A chaperone, of the same gender as the participant, was offered during sample collection and access to the trial zone was restricted to only those researchers and participants involved in the sampling process.

Table 1.5. Number of participants and age ranges for studies involving human volunteers

Study	Number of participants	Age (years)
Chapter 2	24 (12 males & 12 females)	19-26
Chapter 3	10 (all males)	18-32
Chapter 4	1	50
Chapter 5.6	10 (5 males & 5 females)	18-35
Chapter 5.7	10 (5 males & 5 females)	18-59
Chapter 5.8	10 (5 males & 5 females)	18-35

Conditions imposed on the human participants in the various studies are detailed in the relevant chapter describing that study.

1.8 Chemicals

18.2 MΩ-cm deionised water (DI H_2O; Merck Millipore Elix and Element A10, Massachusetts, USA) was used to dilute all samples unless stated. 10 $\mu g.mL^{-1}$ multi-element solutions 1 (MES 1; Ce, Dy, Er, Eu, Gd, Ho, La, Lu, Nd, Pr, Sc, Sm, Tb, Tm, Tu, Y, Yb), 2 (MES 2; Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, No, Ni, Pb, Rb, Se, Sr, Ti, V, Zn), 3 (MES 3; Au, Hf, Ir, Pd, Pt, Rh, Ru, Sb, Sn, Te) and 4 (MES 4: B, Ge, Mo, Nb, P, Re, S, Si, Ta, T, W, Zr), 4 (MES 4; B, Ge, Mo, Nb, P, Re, S, Si, Ta, T, W, Zr) and Hg, obtained from Spex Certi Prep (Middlesex, UK), were used as calibration standards of varying concentrations for all studies where ICP-MS was employed.

70% ultra-pure nitric acid (Romil, Cambridge, UK) was diluted to 2% nitric acid (HNO_3). This 2% HNO_3 solution was used to dilute all samples prior to ICP-MS introduction, and for leaching elements from skin patches in Chapter 2 and 3.

For all studies, accurate weighing of chemicals was performed on a five figure balance (AND, San Jose, CA, USA) and a vortex mixer (Labnet Intl. Inc., Edison, NJ, USA) was used to ensure sample homogeneity. 15 and 50 ml polypropylene centrifuge tubes (Elkay Science, Basingstoke, UK) were used for sample collection, storage and ICP-MS analysis tubes unless stated otherwise.

For the work described in Chapter 3, 15% HNO_3 was used and ethylenediaminetetraacetic acid disodium salt dehydrate (AnalaR, Leighton Buzzard, UK) (EDTA) was diluted with DI H_2O to make up a concentration of 0.1M EDTA. Diluted 1000 $\mu g.mL^{-1}$ indium (Ultra Scientific, North Kingstown, USA) (In) was used as an internal standard to aid element quantification and also to confirm instrument stability. For the removal of impurities from skin patches, 25% tetramethylammonium hydroxide (SigmaAldrich, Gillingham, UK) was diluted to 10% tetramethylammonium hydroxide (TMAH). Also, aqua regia was produced by mixing 3:1 hydrochloric acid (Romil, Cambridge, UK) with 70% ultra-pure nitric acid (Romil, Cambridge,UK).

For the Liquid Skin Pen (LSP) work described in Chapter 5, 0.1M EDTA was used as the sampling solvent for samples that were analysed for elemental content using ICP-MS. 10,000 $\mu g.mL^{-1}$ manganese (Ultra Scientific, N.Kingstown, RI) was diluted to concentrations of 10 $\mu g.mL^{-1}$ and 100 $\mu g.mL^{-1}$ manganese (Mn) and these samples were used in Chapter 5 sections 5.4 and 5.5, respectively. 96% Ethanol (VWR, Leighton Buzzard, UK) was diluted with CHROMASOLV® Plus water (Sigma-Aldrich Company Ltd, Dorset, UK) (H_2O) to produce 50% v/v ethanol ($EtOH_{(aq)}$) used as the sampling solvent for samples that were analysed for VOCs, semi VOCs and non-volatile organic compounds by ESI-MS. 35% ammonia (Fisher Scientific, Loughborough, UK) and 99% acetic acid (Sigma-Aldrich Company Ltd, Dorset, UK) were diluted producing 0.5% v/v ammonia ($NH_{3(aq)}$) and 0.5 v/v acetic acid ($CH_3COOH_{(aq)}$). 50% v/v $EtOH_{(aq)}$, 5% v/v $NH_{3(aq)}$ and $CH_3COOH_{(aq)}$ were used as LSP cleaning solvents.

1.9 Conclusion

The work presented in the subsequent chapters outlines existing and new methodologies to analyse elemental profiles obtained from biological samples of saliva, skin, plasma and urine using ICP-MS, of which is detailed in this chapter. Samples were acquired from participants who experienced psychological, physical and emotional stress after which multivariate analysis in conjunction with descriptive statistics was performed on chemical data to recover potential biomarkers of stress.

ICP-MS and ESI-MS was used to analyse skin samples acquired using the engineered skin sampling device for elements, VOCs, semi VOCs and small molecules. Multivariate analysis and descriptive statistics were also employed to identify biomarkers in specific body sites of the human body. Subsequently, nESI-MS was utilised in order to identify the discovered biomarkers.

2 A study into the effect of psychological stress on the elemental content of human forehead sweat as measured by ICP-MS

Psychological stress is not a condition that can be measured despite the observation of various symptoms of stress such as those mentioned summarised in Chapter 1 section 1.2. Therefore, the study presented in this chapter attempted to identify differences in elemental profiles obtained from human forehead sweat before and after a psychological intervention, a paced auditory serial addition task (PASAT), designed to induce stress. H_0: There was no detectable differences in element concentrations of patches obtained from human forehead sweat from participants that had been subjected to psychological stress i.e. H_0: $E.PS_{PRE} = E.S._{POST}$.

Twenty-four male and/or female volunteers aged between 19 and 26 years participated in the study and 22 complete sets of sweat samples were obtained. Participants attended two sampling sessions each involving an intervention, a neutral and stress session. During the neutral session intervention, participants were asked to sit comfortably and listen to eleven minutes of classical music. In contrast, during the stress session intervention, participants were asked to undertake a mental arithmetic test (PASAT), chosen to provoke stress. Interventions, eleven minutes long, were sandwiched in between cognitive function assessments that did not induce stress, and were conducted as part of another study into the effect of stress on cognitive function. Sampling sessions ran in the morning before which the participants had fasted for a minimum of nine hours. During this time, participants were able to drink only water and refrained from washing or using oral hygiene products on the morning of their visit. In each session, two 30 minutes sweat samples were collected (one each for elemental analysis and VOC analysis) from the participant's forehead. The first sweat sample was collected before the experimental intervention (neutral/PASAT) and the second following the completion of the intervention.

The data presented in this chapter is part of a wider study, devised by Matthew Turner (personal communication) in collaboration with the author and Helen Martin (personal communication), referred to in Appendix VII Publications reference 2. The PASAT was introduced to the experiment by Dr Stephan Bandelow (personal communication) and Dr Louisa Edwards (personal communication). The author, Helen Martin and Matthew Turner collected all VOC and elemental skin samples as well as VOC breath samples and cardiovascular readings. Assistance in sample collection was also given by Aditya Malkar,

Toni McKay and Elliot Poole. All elemental skin patch analysis and subsequent data analysis of the results was carried out by the author.

2.1 Experiment Outline

Figure 2.1 shows the experiment outline described here.

2.1.1 Physiological Measurements

Throughout the experiment, cardiovascular responses, including sixteen heart rate (HR), systolic and diastolic blood pressure (BP), were monitored and recorded from each participant on each visit using a Datex-Ohmeda S/5™ monitor (Instrumentarium Corp, Finland). Four initial measurements were taken to provide a baseline and twelve other measurements were taken at key pre-defined times during each session (Figure 2.3). Also taken were continuous galvanic skin response (GSR) measurements using a Sensewear™ WMS (APC Cardiocascular Ltd, UK) armband. The adjustable armband was placed around the right bicep of the participant. The device contained two electrodes that made contact with the skin allowing the measurement of electrical resistance across the sampling area. This related to moisture levels at the skin surface and the data collected was recorded on the device after which the results were downloaded to a computer following the completion of the sampling stage of the study. The device contained a button for time stamps, which was pressed at key stages during the experiment (Figure 2.3).

2.1.2 Cognitive Function Test Battery

Subjects completed four computer based cognitive function tests prior to and following the intervention designed to measure reaction time and concentration levels. Shown below are the tests in the order they were taken as well as a brief description about each of them.

1. *Stroop test* Duration: Approximately 3.5 minutes

A psychological test measuring the mental vitality and flexibility of a subject. Humans have an ability to read words more quickly and automatically than they can name colours. This direct attention task involved the subject having to manage their attention, inhibit or stop one response in order to say or do something else. This is performed by words typed in different colours to what they are on a computer. For example; the word “blue” is written in green ink: “blue”.

2. *Flanker test* Duration: Approximately 3.45 minutes

A test of attention in the presence of distracting information. Subjects were asked to always respond as quickly as possible, whilst avoiding errors, in this reaction time task. An example

of the Flanker test is an array of five symbols where the central symbol is always an arrow, which points either horizontally left or right. If the central arrows points to the left, the subject would press the left arrow key, if it points to the right, the subject should press the right arrow key. The four symbols on either side of the central arrow are called flankers. These can be arrows, sometimes pointing in the same direction as the middle arrow. Sometimes the flankers point in the opposite direction to the target. For example: if the arrow set visible is →→←→→, the participant should press the left arrow key (←) as quickly as possible.

3. *Sternberg test* Duration: Approximately 3.5 minutes

A test of memory storage capacity. Subjects were instructed to focus on a point on a computer screen where lists of words appear. In this task each word appears on the screen, one after another, erasing out before being replaced by the next word. For successive trials, the list is variable in length and a new target (the word) appears. It is important to note that this target may or may not have been present in previous lists. When a word appears the subject must press one button and a different button when the new word appears in the list. Feedback is given on the correctness of the decision when the button is pressed, and then the next list is presented. The general idea is that the longer the word list, the longer it takes the subject to recall whether the target word has appeared previously.

4. *Rapid Visual Information processing test (RVP)* Duration: Approximately 6.5 minutes

A test of sustained attention requiring working memory for its successful execution. Single digits are presented in quick succession on the screen, and when a target sequence is identified a button must be pressed. A random series of numbers is presented in single digits, each digit disappearing before the next. A target sequence, such as 3 followed by 5 then 7, must be identified within the random sequence. For example, on the screen the following numbers appear one at a time 1, 3, 5, 8, 5, 7, 6, 4, 3, 5, 7, 2, 3, 4, 5, 7, 3, 5, 7. The subject should identify that the target sequence appeared twice within the random numbers sequence by tapping the spacebar on the computer keyboard following correct recognition of the target sequence.

2.1.3 Inducing Psychological Stress: The PASAT

The paced auditory serial addition test (PASAT) was first developed to assess the effects of traumatic brain injury on cognitive functioning by Gronwall and Sampson in 1974 by measuring the speed of information.[79] However, the PASAT is now known as a measure of multiple functional domains, requiring successful completion of a variety of cognitive functions, principally related to attention. The PASAT creates an excessive amount of anxiety

and frustration in participants which affects their performance on this test and has frequently been used as a psychological stressor in several recent studies[80–82].

A series of digits are presented at two second intervals via an audio feed. The test requires subjects to verbally respond the arithmetic sum of a new digit with a number immediately preceding it. For example, the participant would verbally answer the following answers (in brackets) if the audio feed supplied the list of numbers 2, 7 (**9**), 4 (**11**), 6 (**10**) etc. In this study, the PASAT was used to induce psychological stress. Incorrect answers, or at pre-defined places during the PASAT, were emphasized with the use of a loud horn to confuse and further apply stress to the participant. The use of the horn was designed to frustrate and disorientate the participant as it often overlapped with the next number causing the errors in the following answer. Detailed instructions of the PASAT can be found in Appendix V as well as the PASAT answer sheet (Figure 8.4).

Although the PASAT has demonstrated good psychometric properties such as test-retest reliability and internal consistency, there are issues that should be considered when administering and interpreting this test. The test is susceptible to practise effects and is negatively affected by low math ability, low IQ and increasing age. Those with speech or language impairments, as well as those who naturally speak slow for cultural and geographical reasons, are at a distinct disadvantage due to the demands for rapid responding placed on the subject.

In addition to the stress condition, participants were asked to attend the lab for a placebo condition on a separate visit on a different day. The placebo condition involved the subject listening to classical music for eleven minutes in a quiet room, where their blood pressure and heart rate was again monitored.

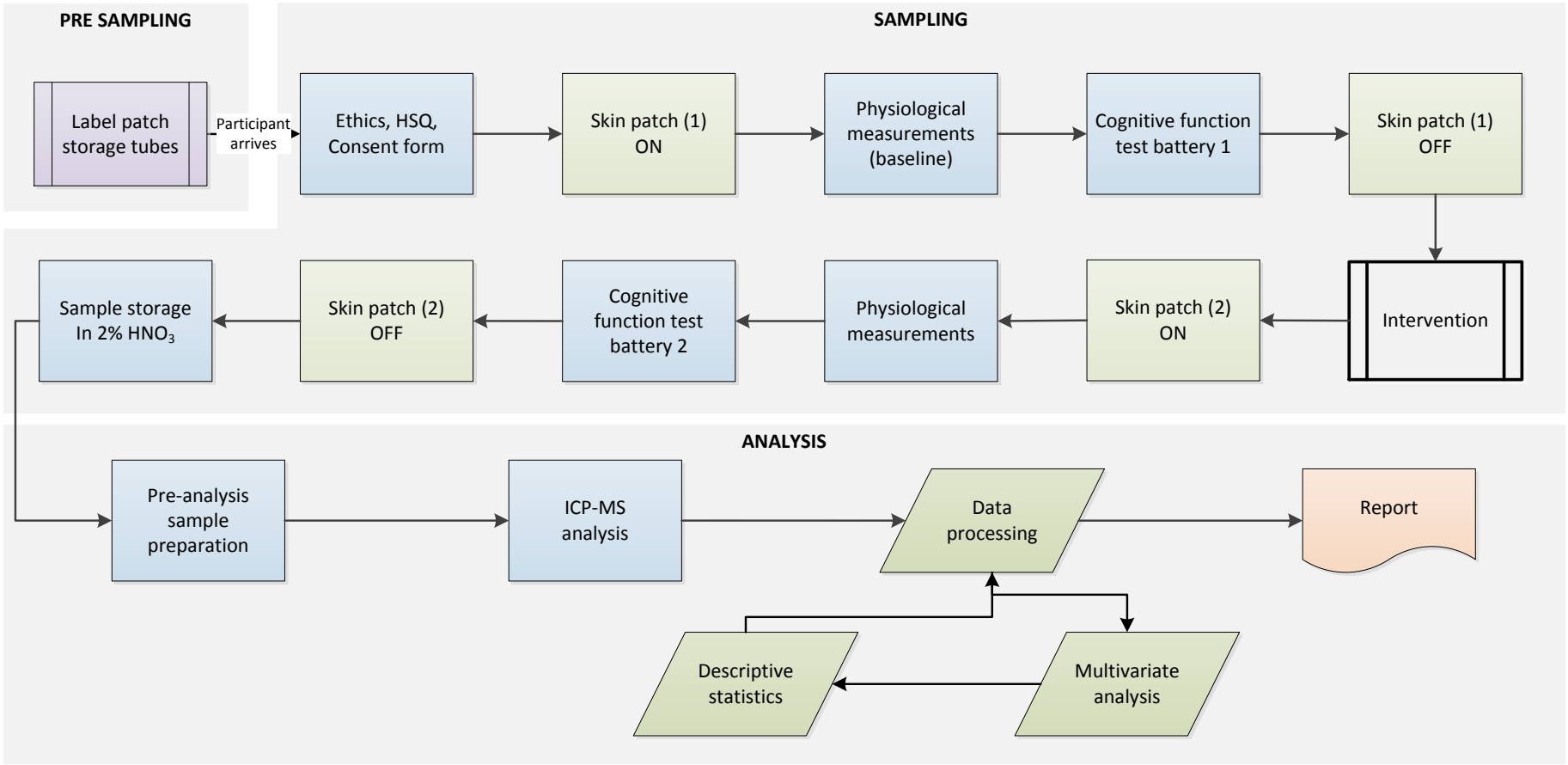


Figure 2.1. Experiment outline for elemental sweat collection and analysis obtained by psychological stress

2.1.4 Skin Patch Sampling Area

Sweat glands create a minute cooling effect on the skin, an effect that increases along with the perspiration rate. The forehead is one of the most visible anatomical areas of the body that features the highest concentration of sweat glands.[83]

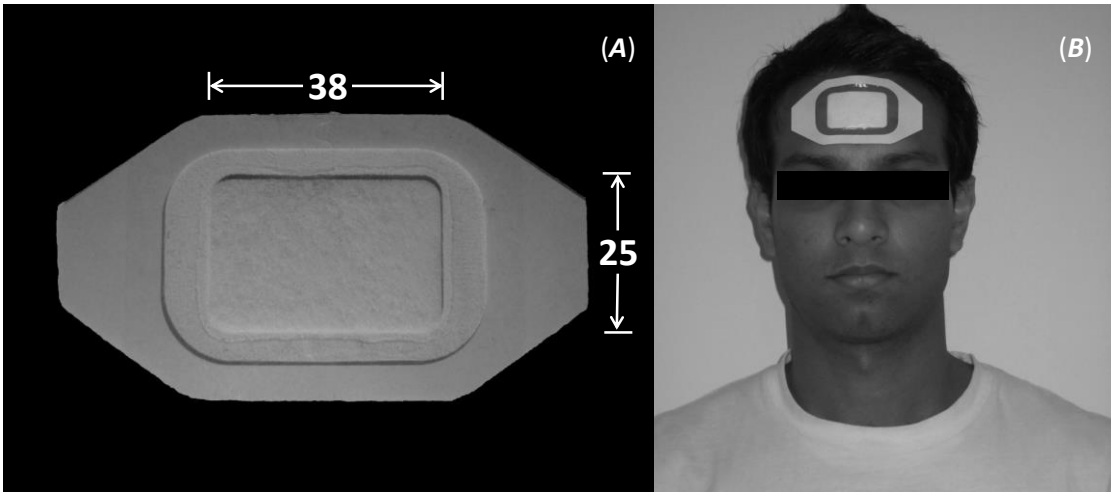


Figure 2.2. Skin patch. A: 3M™ Tegaderm™ +pad film dressing with non-adherent pad. B: Skin samples were collected by sticking the patch to the middle of the participant's forehead

Salvesen investigated the amount of sweating in the lateral (lateral angle of the eye) and medial sites (root of the nose) in the forehead after body heating, and, at another occasion, after intracutaneous injection of a drug (inducing emotional stress). Salvesen found there was an increase in the level of sweating in the lateral site compared to the medial site during body heating but nearly all symmetrical sweating responses were observed after the injection.[84]

Although several studies have indicated the axillae and palms as locations for monitoring emotional sweating as a consequence of mental strain, due to accessibility issues as a result of other sampling constraints, in this study the forehead chosen as the sampling area. Previous literature, outlined in Chapter 1 section 1.2, exhibited a correlation between increased forehead sweat and emotional stress response allowing an estimation of the degree of emotional stress on humans.[11, 12]

For each session skin elemental samples were collected using two 3M™ Tegaderm™ +Pad film dressing with non-adherent pad (3M™, UK). The pad (size 38mm x 25mm) was bonded to a larger film backing (size 70mm x 50mm) with a non-latex, hypoallergenic adhesive. These patches are typically used as film dressings with a non-adherent pad that is waterproof, bacterial and viral barrier. As well as being relatively cheap, the cotton pads have been chemically proven to be super absorbent and remain intact without leakage,

unlike frequently used patches containing cotton gauzes. Patches remained sealed in the sterile packaging until the moment prior to placing on participant forehead.

2.1.5 Skin patch sampling

A total of four samples were collected from each participant (two samples from two visits). Samples were obtained from the centre of the forehead with a sample collection time of 30 minutes (Figure 2.2). The first sample, ("Pre") was obtained prior to the intervention and the second ("Post") was obtained immediately following the intervention. Upon removal from the forehead each patch was placed into a 50 ml polypropylene vial with a screw top and stored at 4°C until analysis.

2.1.6 Sample Analysis

On the day of analysis, the cotton pad of the 3M™ Tegaderm™ +Pad was then extracted off the adhesive with plastic tweezers and placed into 25ml of 2% HNO_3. This tube was left on a shaker (ELMI Skyline Shaker, Riga, Latvia) for the duration of 60 minutes at a tilt of 5°. The pad was removed from the 2% HNO_3 using plastic tweezers and disposed of appropriately. The remaining solution in the tube, containing leached elements from the pad, was then sealed ready for analysis. Ninety two participant skin samples were analysed using ICP-MS (Chapter 1 section 1.3.5) in ascending participant ID order (Supplementary information Table SI.2.2). Pre and post intervention sample data was grouped in to perform MVA followed by the conductance of significance testing to discover whether any elemental differences between samples existed.

2.2 Results and Discussion

Elemental analysis was performed on a total of ninety eight patches. These included ninety two sampled skin patches and six blank patches. Of the ninety two patches, eighty eight formed twenty two completed sample sets obtained from ten male and twelve female participants. Psychological stress scores were also determined, using a newly designed formula, using cardiovascular responses that were collected from each of the sessions participants attended.

2.2.1 Cardiovascular response

Cardiovascular reactivity is defined as the change in BP, HR and other haemodynamic parameters in response to physical or mental stimuli.[85, 86] The aim of these efforts was to measure HR and BP changes under psychological stress conditions, and compare them to baseline values.

	End Phase	All equipment removed. Participant booked in for second (and final) visit Samples logged. Breath and skin VOC samples transferred to 4°C for storage **Skin patches removed & sealed in storage tube (2)** Task evaluation questionnaire filled out for cognitive function tests Breath mask removed
+11 - +41 mins	Post-intervention cognitive	RVIP (~6.5min). HR and BP taken after the test. STERNBERG (~3.5min). HR and BP taken after the test. FLANKER (~2.45min). HR and BP taken after the test. STROOP (~3.5mins). HR and BP taken after the test.
	Post Intervention Measurements	Breath sampling started SCM Breath mask fitted & sampler turned on **Skin patch (2) applied to forehead**
+0 - +11 mins	Intervention	INTERVENTION (PASAT OR PLACEBO)
	Transition phase A	SCM Samples logged. Breath and skin VOC samples transferred to 4°C for storage **Skin patch removed & sealed in storage tube (1)** Task evaluation questionnaire filled out for cognitive function tests Breath mask removed
- 30 - 0 mins	Pre-intervention cognitive function tests	RVIP (~6.5min). HR and BP taken after the test. STERNBERG (~3.5min). HR and BP taken after the test. FLANKER (~2.45min). HR and BP taken after the test. STROOP (~3.5mins). HR and BP taken after the test.
	Baseline Measurement	Pressed time stamp button on SCM and recorded time Breath mask fitted & sampler turned on Time started and recorded **Skin patch (1) applied to forehead** Forehead cleaned witi deionised water Skin conductivity meter (SCM) attached to upper right arm (electrodes in contact with skin) Resting Bp Resting HR
	Introduction and Briefing	Consent Form Health screen questionnaire & Psychology questionnaires Participant info sheet

Figure 2.3. Psychological stress sample collection timeline

From each participant, HR, systolic BP (P_S) and diastolic BP (P_D) were collated and the average observations show the cardiovascular responses to the PASAT and neutral interventions. Figure 2.4 compares the differences in the observed cardiovascular response during the stress (PASAT) and neutral (placebo) sessions.

The psychological stress score (PSR) equation was devised by Matthew Turner (personal communication). Individual cardiovascular responses to the PASAT and neutral interventions were scored on the basis of observed changes in their systolic BP and HR. An average of the first three baseline observations were used to give BP and HR start values (${P_S}^{start}$and HR^{start}). The maximum observed values of the four readings taken during the intervention period were assigned points of maximum 'stress'. Equation 2.1 defines a psychological response score for both the PASAT (PSR_S) and neutral interventions (PSR_N):

$$PSR = \frac{\Delta P_S}{{P_S}^{start}} \; X \; \frac{\Delta HR}{HR^{start}} \; X \; 1000$$

where $$\Delta P_S = {P_S}^{max} - {P_S}^{start}$$ (Equation 2.1)

and $$\Delta HR = HR^{max} - HR^{start}$$

The PSR was formulated to highlight relative change between the cardiovascular measurements taken by assessing i) the relative change in systolic BP (ΔP_S) compared to baseline observations (${P_S}^{start}$) and ii) the relative change in HR (ΔHR) compared to baseline observations (HR^{start}). The scores were framed to give a higher score to participants who presented a larger cardiovascular response during the interventions. Scores are summarized in Table 2.1 and Figure 2.4 shows the differences in response between males and female participants.

ANOVA was performed in an attempt to observe whether any statistically significant differences were present between the stress and neutral responses (Appendix Table 8.2). The results show that significant differences (95%) were observed between the two sessions for all participants due to a higher calculated F value (22.825) compared to the theoretical critical F value (4.073). All participants, except one, produced a higher stress response during the stress intervention compared to the neutral intervention. Participant 4 produced a stress response of 0.9 and 5.3 for the stress and neutral sessions, respectively, suggesting the participant was not affected by the PASAT intervention. The average response of the other 21 participants was 64.7 ± 20.9 for the stress session and 4.4 ± 1.8 for the neutral session. There was no statistically significant evidence of a difference between male and female

stress scores as a calculated F value of 1.435 was attained, which was smaller than the critical F value of 4.351. However, female participants produced a larger range of responses for the stress session (PSR = 12.9 to 233.9) compared to male participants (PSR = 11.1 to 149.9). Furthermore, for the neutral session, female participant stress responses (PSR = 0.8 to 7.7) exhibited less variability than male stress responses (PSR = 0.1 to 18.6).

The data indicates that although male and female participants have similar baseline stress scores, following the intervention, female participant stress increased larger than male stress scores proposing the presence of gender differences in stress.

2.2.2 Galvanic Skin Response

Galvanic skin response (GSR) is a change in the electrical resistance of the skin caused by emotional stress. GSR is the phenomenon that the skin momentarily becomes a better conductor of electricity when either external or internal stimuli occur that are physiologically arousing. However, it must be mentioned that arousal refers to the activation and is considered to be a dimension of emotional response but arousal is therefore not the same as measuring emotion, but is an important component of it.

Electrical resistance, associated with skin moisture levels, was collected from participant 12 for both the stress and neutral sessions (Figure 2.5). An initial spike can be observed in both sessions. The response may be recognised for a nervous participant whilst being briefed on the sampling procedures and tasks during the visit.

Alternatively, it may be the device equilibrating once it switches itself on. On both visits, this response decreased from an initially high reading following the start of the first cognitive battery as the device stabilised. During the intervention the GSR for the stress session increases just after the start of the PASAT proposing the participant to be under pressure from the test whilst the GSR remains stable at the baseline during the placebo intervention. During the PASAT the GSR then begins to drop back towards the baseline prior to the intervention although it stabilises above the pre intervention baseline. This stabilised post intervention GSR level, at a higher level than prior to the PASAT intervention, is a sign that the body has been through stress. During the placebo intervention, no change is observed in the galvanic skin response.

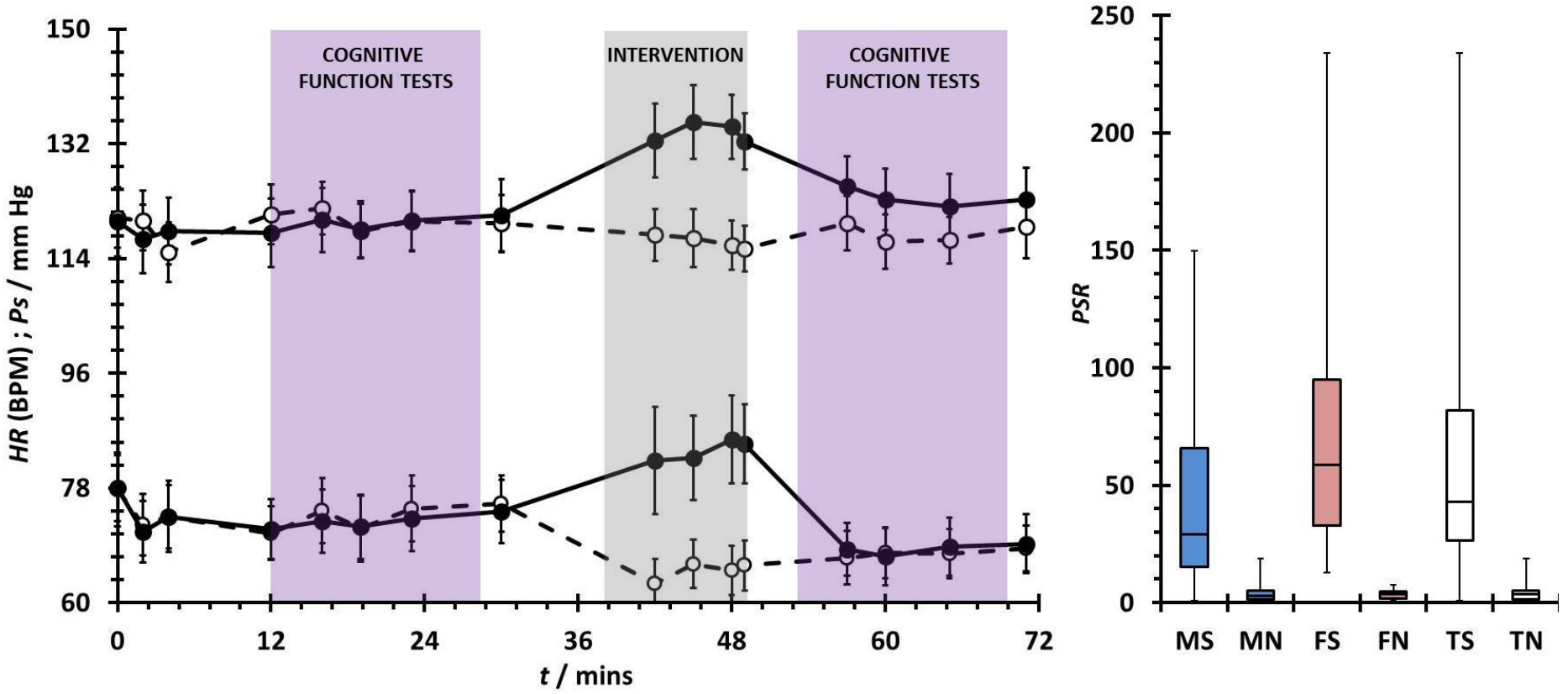


Figure 2.4. Mean responses of 22 participants for systolic blood pressure (P_S, top), and heart rate (*HR*, bottom) over the 70 min stress (solid line) and neutral (dashed line) sessions. The error bars designate the 95% confidence limit of the mean. MS = male stress, MN = male neutral, FS = female stress, FN = female neutral, TS = total stress, TN = total neutral

Table 2.1. Cardiovascular response scores of participants during the neutral (PSR_N) and stress intervention (PSR_S). HR^{start}: Baseline heart rate recorded at start (BPM); HR^{max}: maximum heart-rate (BPM); P_S^{start}: baseline systolic blood pressure recorded at start (mmHg); P_S^{max}: maximum systolic blood pressure (mmHg) and PSR: Stress response score, see Figure 2.4.

Ref.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
M/F	M	F	M	M	M	F	F	M	F	M	M	F	F	F	M	M	F	F	F	F	F	M
	PASAT intervention																					
HR^{max}	90	119	70	92	80	77	105	91	90	90	96	100	82	134	95	84	146	66	97	95	105	85
HR^{start}	78	94	54	90	69	68	81	76	66	73	59	66	72	84	75	76	88	42	82	61	65	54
P_S^{start}	141	121	112	136	121	113	105	120	129	141	113	119	102	117	107	138	112	112	116	126	110	144
P_S^{max}	152	148	122	142	144	124	125	136	155	168	129	138	117	146	121	153	152	120	133	154	132	162
PSR_S	11.6	60.6	27.5	0.9	30.3	12.9	56.7	26.1	74.2	44.8	87.6	84.5	19.7	149	34	11.1	233	40.8	27.5	125	125	72.6
	Neutral intervention																					
HR^{max}	81	102	65	72	74	83	98	85	69	101	71	89	101	73	82	95	113	54	79	94	71	70
HR^{start}	75	90	58	65	68	79	85	82	61	81	60	72	84	56	73	78	81	49	60	79	68	56
P_S^{start}	126	120	125	118	120	107	102	125	120	135	113	113	111	116	107	133	118	108	120	119	103	147
P_S^{max}	127	121	125	124	122	122	101	118	123	138	122	111	109	123	114	131	117	107	122	116	105	148
PSR_N	1.0	0.8	0.1	5.3	1.6	7.4	1.9	2.0	3.2	4.7	15.5	5.0	4.2	18.6	7.7	3.9	2.7	1.2	4.5	4.5	1.0	1.4

Table 2.2. Mean concentration (ng.mL^{-1}) and relative change (R_C(%)) between pre and post skin patches for the stress and neutral interventions. In brackets, relative standard deviation (%RSD) for each sample set (for blank n = 6, samples n = 12)

Sample	Na	Zn	S	Sn	Ca	K	Ba	Mg	Ti	Cu
	μ									
Blank	7100 (7.25)	248 (3.3)	216 (2.28)	168 (7.53)	65 (91)	29 (87)	26 (4.32)	2.7 (87)	1.7 (2.13)	0.0 (0.0)
Pre Stress	2198 (127)	178 (67.1)	139 (74.4)	49 (143)	220 (122)	80 (159)	6.9 (132)	8.3 (158)	2.0 (241)	1.5 (259)
Post Stress	1906 (116)	160 (53.2)	135 (70.0)	37 (146)	133 (135)	29 (104)	5.9 (120)	5.3 (195)	1.0 (51)	1.5 (305)
Pre Placebo	2385 (120)	167 (58.5)	154 (84.5)	44 (137)	126 (114)	92 (282)	7.2 (132)	5.6 (129)	1.0 (53)	1.4 (326)
Post Placebo	1505 (162)	180 (59.5)	154 (83.7)	29 (203)	184 (146)	32 (130)	4.9 (163)	5.4 (141)	1.1 (57)	1.5 (313)
	R_C (%)									
Stress	-13.3	-10.2	-2.59	-23.9	-39.7	-64.1	-13.5	-35.8	-52.3	-2.36
Placebo	-36.9	7.36	-0.36	-34.5	46.6	-65.5	-31.9	-3.70	4.96	3.89

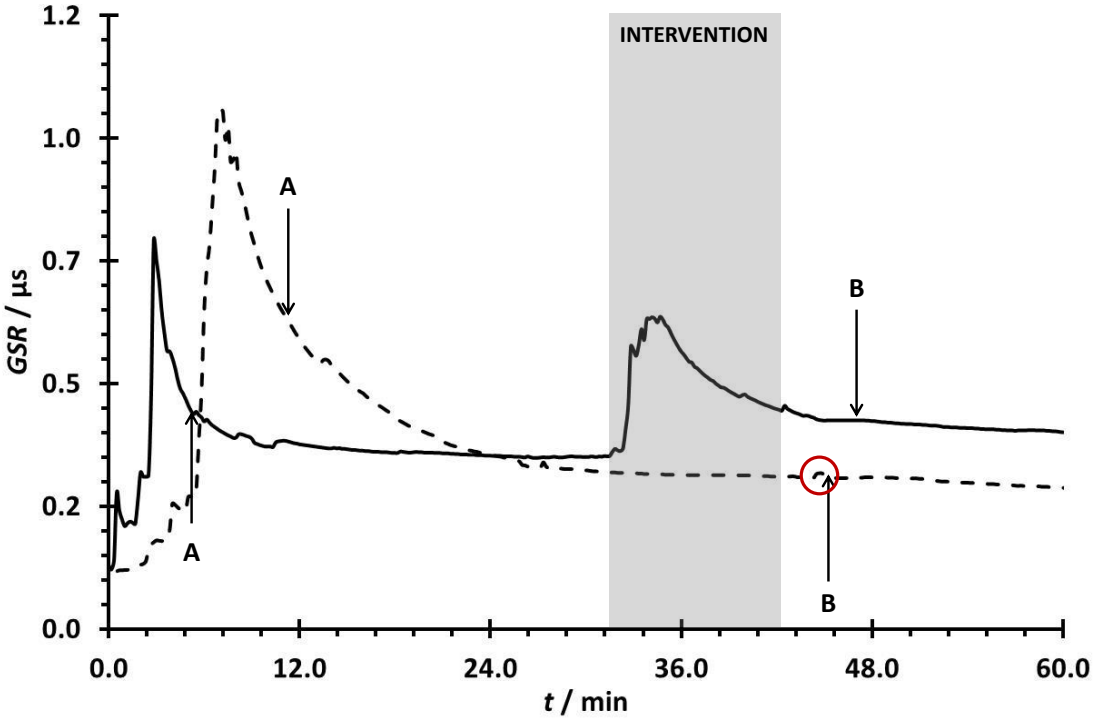


Figure 2.5. Galvanic skin response (GSR) of participant 12 during the stress (solid line) and neutral (dashed line) sessions where (A) indicated the start of the pre intervention cognitive battery and (B) indicates the start of the post intervention cognitive battery

The cardiovascular and GSR measurements recorded during this study prove the PASAT to cause change in autonomic nervous system response compared to the neutral session demonstrating that the PASAT was an effective stressor. The GSR produced a response at the skin surface during the stress intervention, proposing that the elemental profile of skin may also change during this time.

2.2.3 Elemental profiling of skin by ICP-MS

A total of ninety eight patch samples were analysed including six blank patch samples. Ninety two of these patch samples were skin samples from twenty four participants. From these twenty four participants, twenty two provided complete sample sets (two skin patch samples each for both the stress and placebo sessions). Forty four skin patch samples were collected from the placebo sessions and forty eight skin patch samples were collected from the stress sessions as participants 9 (labelled E11) and 23 (labelled 25) were unable to attend a second session which in this case was the placebo sessions. Consequently, data presented in this chapter excludes results attained from the non-complete participants.

Each sample was analysed once. An external calibration sample set, to semi quantify element concentration, was analysed at the start of each analysis sequence. From the forty elements that were analysed, ten were detected in blank and participant skin patch samples

(Table 2.2). Very large elemental concentrations in the skin patches were detected for Na with approximately 2000 $ng.mL^{-1}$. Over ten times smaller than Na concentrations were discovered for Zn, Ca and S, which were found to have average concentrations of approximately 170, 165 and 145 $ng.mL^{-1}$, respectively. Traces of Ba, Mg, Cu and Ti were also detected. Relative changes (R_C (%)) were also calculated for each of the detected elements (Table 2.2). All elements obtained from the stress intervention decreased in concentration from the pre to the post intervention patches. For the neutral intervention only six of the ten elements decreased in concentration from the pre to the post patches whereas four elements increased in concentration (Figure 2.6). Large relative concentration changes between pre to post intervention patches, in both the stress and neutral interventions, were observed for K. In both sessions K concentrations decreased by approximately 65%. In the stress session a large decrease of approximately 52% was also observed for Ti whereas in the neutral session Ti concentration increased by nearly 5%. Large decreases in elemental concentrations were also detected for Ca, Mg and Sn whilst decreases were also identified for Ba, Na, Zn, Cu and S in the stress session. In the neutral session, large decreases were observed for Na, Sn and Ba whilst smaller decreases were detected for Mg and S. However, in this neutral session, the elemental concentration of Ca increased between the pre and post intervention by almost 50% and small increases were detected for Zn, Ti and Cu.

Table 2.3. Two-way ANOVA (with replication) for elemental sweat samples obtained before and after each intervention

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between pre and post stress samples						
Sample	219922	1	219922	0.379	0.538	3.862
Columns	160918023	10	16091802	27.765	1.55E-41	1.851
Interaction	836238	10	83624	0.144	0.999	1.851
Within	267762402	462	579572			
Total	429736585	483				
Between pre and post neutral samples						
Sample	788079	1	788079	1.202	0.274	3.862
Columns	144334339	10	14433434	22.012	1.134E-33	1.851
Interaction	7816626	10	781663	1.192	0.294	1.851
Within	302936926	462	655708			
Total	455875970	483				

Table 2.2 also shows the large relative standard deviations obtained for elemental concentrations observed in sampled forehead patches. This was initially due to variable elemental concentrations in the patches themselves prior to forehead sampling and then different elemental profiles from participant foreheads themselves.

ANOVA was conducted for skin patch samples obtained between both stress and neutral sessions (Table 2.3). Between pre and post PASAT intervention samples the observed the calculated F value (F_{calc}) value of 0.144 (interaction) was smaller than the critical (F_{crit}) value of 1.902. Between pre and post neutral intervention samples the F_{calc} value of 0.294 (interaction) was smaller than the F_{crit} value of 1.902. Due to $F_{calc} < F_{crit}$ it can be deemed that there is no evidence of statistically significant differences (95%) between pre and post samples for both interventions and any differences can be attributed to random errors. Intriguingly, when grouping pre intervention samples and comparing them with post intervention samples from both interventions, ANOVA showed evidence of significant differences (95%) resulting in F_{calc} (22.825) > F_{crit} (4.073) (Table 8.2). However, it must be noted that participants conducted these interventions on different day with different conditions (e.g. different room temperature). In order to explain why differences may have been seen by grouping all pre and post samples, elemental breakdown of patches must first be scrutinised.

Figure 2.6 explains why elemental differences were not detected between the pre and post intervention skin patch samples for both the stress and neutral sessions. For five of the ten elements detected, concentrations observed in blank patches were much higher than skin patches obtained from participant foreheads. Table 2.2 shows that Na concentration in blank patches were found to be over 4.5 $\mu g.mL^{-1}$ higher than the mean concentration obtained between the pre and post participant sample patches, for both the stress and neutral interventions. Sn, Zn and S concentrations in the blank patches are also high compared to those obtained from the participant skin samples, with an average concentration of 119, 68 and 62 $ng.mL^{-1}$, respectively. There are only small differences for Ba, Ti, Cu and Mg between the blank patches and those obtained from the skin whereas the concentrations obtained for K and Ca in the participant skin patches are higher than those in the blank patches, with concentrations of approximately 60 and 150 $ng.mL^{-1}$, respectively. Pre and post intervention Ca and K changes are significant, as, unlike the other elements, these are not drowned by the high elemental concentrations found in the blank patches.

The elements that decrease in concentration, from the blank patches, when placed onto the skin may indicate that they are being absorbed into the skin from the patches. Na decreases in concentration so drastically that it is either transferred onto or into the skin upon contact with the patches.

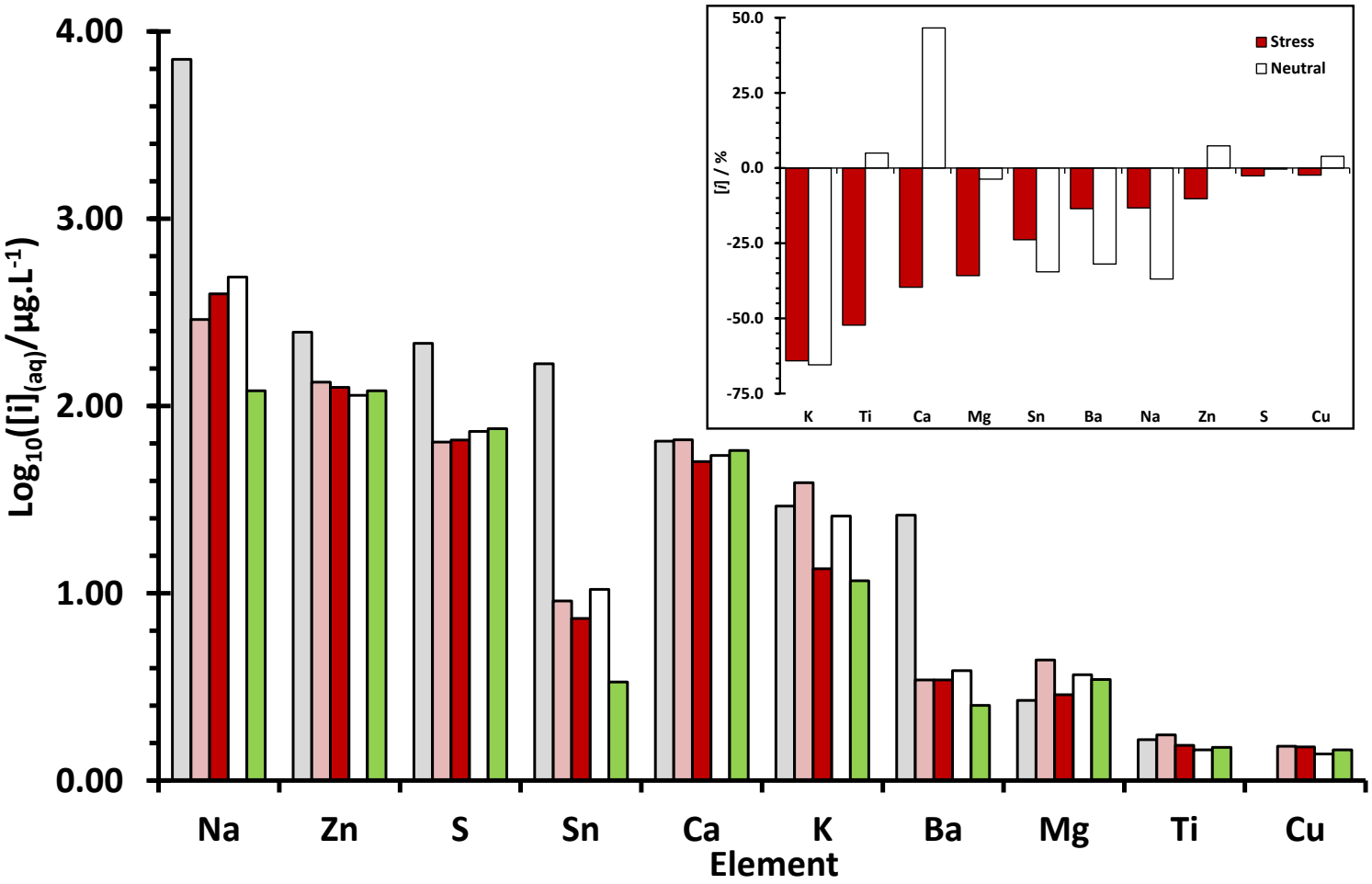


Figure 2.6. Concentration of detected elements from sweat patches: blank (grey), pre stress intervention (pink), post stress intervention (red), pre neutral intervention (white) and post neutral intervention (green). Insert: relative change (%) in concentration between pre and post stress intervention (red) and pre and post neutral intervention (white) patches

2.2.4 Multivariate Analysis

Due to high elemental concentrations in the blank patches, MVA was carried out as a training exercise rather than a tool to identify potential biomarkers of psychological stress.

The skin data set consisted of concentrations (ng.mL^{-1}) of the 10 element skin components (variables) against the participants (observations).

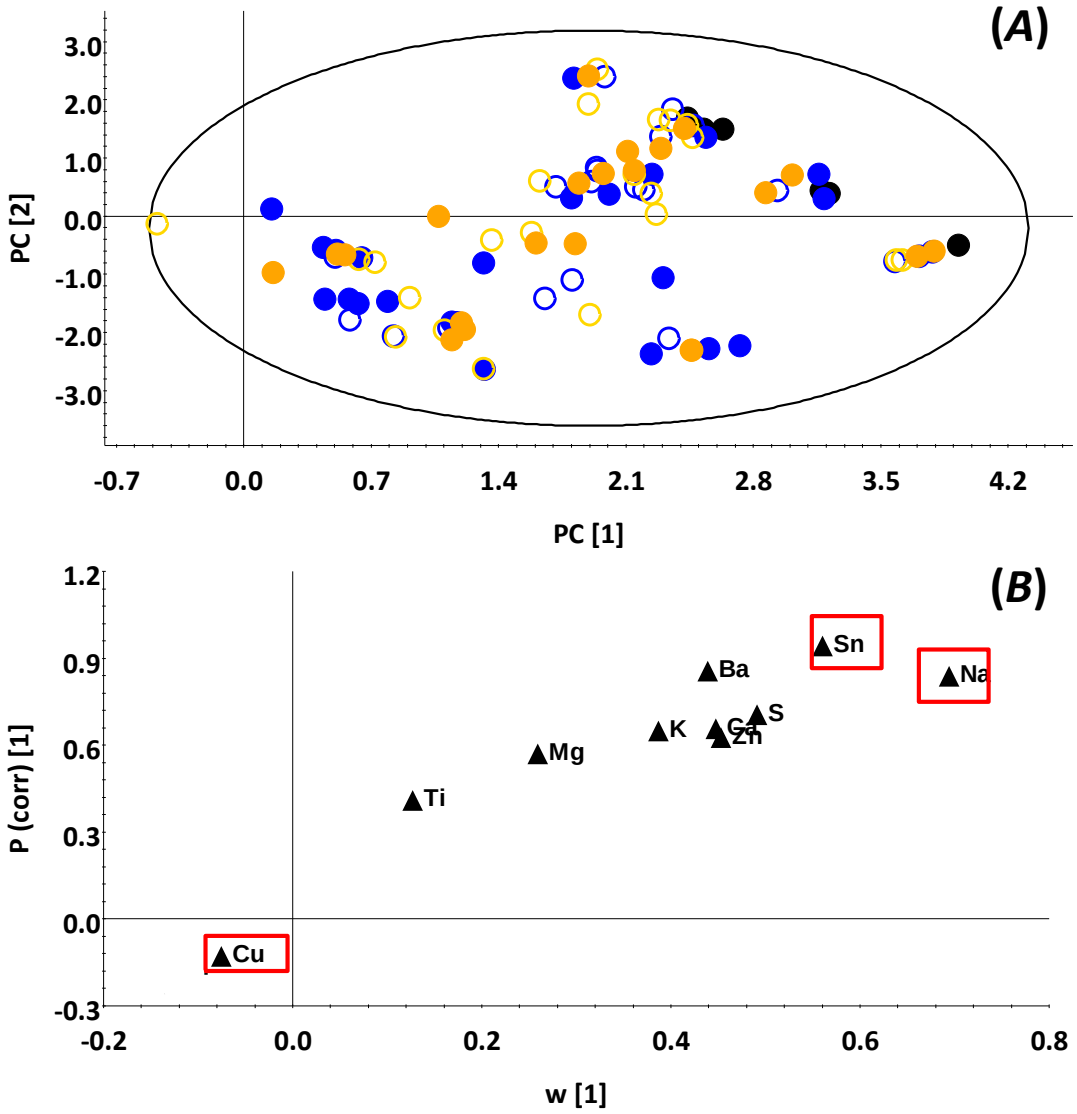


Figure 2.7. Multivariate analysis (MVA) using blank (black), pre neutral intervention (blue circles), post neutral session (blue dots), pre stress session (orange circles) and post stress session (orange dots) sweat patch samples. Unsupervised PCA of three elements (A) identified from the S-plot generated from the modelled covariance (P(corr)[1]) of the PLS-DA from the concentrations (ng.mL^{-1}) of 10 skin components (variables) from 98 skin patch samples (observations). The three variables highlighted (red squares) were found to provide the highest magnitude (concentration) of the variables in the data set and greatest reliability (P(corr)[1] axis) of the variables in the data set (B).

From the ten elements detected by ICP-MS, three elements were selected from the s-plot (Figure 2.7 B) created from the PLS-DA model. Using these three variables, PCA was performed (Figure 2.7 A). However, PCA did not appear to enable elemental separation

between pre and post forehead sweat sample following either intervention. The lack of separation was due to the high elemental concentrations observed in the blank patches.

2.3 Conclusion

Ten elements were detected from forehead sweat using the 3M™ Tegaderm™ +Pads. However, analysis of the blank patches revealed high concentrations of those elements detected. Although it may be interesting that K concentrations decreased following both interventions, comparing the elemental concentrations in the blank patches to those obtained from participant forehead patches revealed that concentrations of elements decreased in at least eight of those elements. These large concentration decreases could be due to absorption of elements from the patches onto the forehead sampling area, through forehead pores and into the body. Alternatively, the elemental variability may be due to the unknown element amount in the patches themselves. In the blank patches, large concentrations of K, Zn, S and Sn were found, whilst there were also relatively large concentrations of Ca, K and Ba in comparison to the small amount of Mg, Ti and Cu. Consequently, the results presented in this chapter cannot be used to justify elemental markers of psychological stress. Also, Ni could not be accurately analysed due to the instrument analysis cones used which contained Ni material. In future studies, Pt cones should be used if Ni was to be analysed. However, during the analysis of this experiment no Pt cones were available.

Although the elemental concentrations are observed at varied levels in the blank patches and the participant patch samples, further washes should be conducted to remove traces of impurities off the patches. In order to accurately quantify the concentration changes between the interventions, the blank patch elemental concentrations should ideally be as little as possible. The patches could be subjected to a series of washes to reduce the level of any acidic, basic and ionic components. Alternatively, different patches could be sought.

Heart rate, blood pressure, galvanic skin response and breath measurements did prove that the PASAT intervention used in this study induced psychological stress.[87]

Despite this study proving unsuccessful in determining the elemental markers of psychological stress, future experiments should incorporate similar patches that have been elementally purified in order for any changes in elemental concentrations to prove statistically valid. Further to the use of elementally purified patches, in order to repeat this study again in the future, that is to study the effect of psychological stress on the elemental content of human forehead sweat, participants who have not performed the PASAT will be required. This is due to removing any possible memory effects of the intervention which may provide a bias for participants.

3 The effect of physical stress on elemental content in human plasma, skin and urine and the effect on elements, volatile organic compounds and small molecules in human saliva

There are numerous types of stress which are believed to exist yet there is little evidence to chemically measure stress in the public domain. As stated in Chapter 1 section 1.2 there are a number of physical symptoms associated with the condition but few can be measured. A form of stress thought to produce the strongest symptoms is known as physical stress where the body reacts to physical exertion.

The experiment in this chapter sought to identify differences in elemental profiles from a variety of biological human samples following an intervention designed to induce physical stress. Elemental profiles were obtained by ICP-MS analysis from a number of samples, including skin, saliva, urine and plasma. H_0: there was no detectable difference in biological state following induced physical stress in human participants i.e. H_0: $PS_{REST} = PS_{STRESS}$. These biological samples were identified for the research presented in this chapter as they covered the major aspects of human biological pathways, which may chemically change when undergoing stress.

Further to this, data collected from samples analysed by the same method were to be stitched together to produce one data set that would enable successful MVA to be performed and to identify any major elemental contributors from modelling of this super set of data. Finally, data from saliva samples, obtained from participants and analysed by three different techniques, was to be combined using a similar method in order to identify major biomarkers of stress.

The experiment in this chapter was devised and carried out by the author (primary investigator) in collaboration with Matthew Turner (personal communication), Helen Martin (personal communication) and Aditya Malkar (personal communication). Experiments were conducted in Prof Ronald Maughan's laboratory in the School of Sports Exercise and Health Sciences (SSEHS) under the supervision of Dr Philip Watson (personal communication), who also collected blood from participants. The author was the primary investigator responsible for the participant's welfare and collected all physical measurements, skin samples from the participant's forehead, skin conductivity measurements, saliva samples, rate of perceived exertion data and supervised the stress intervention. Matthew Turner collected all breath and BioVoc samples whilst Helen Martin prepared skin patch samples prior to sample collection, collected cardiovascular data and assisted the author with the stress intervention.

Urine was provided by the participant with no aid and blood samples were collected by Dr Philip Watson. All samples were separated and prepared for storage by Aditya Malkar. Plasma, saliva, skin and urine samples analysed by ICP-MS was performed by the author whilst saliva samples for VOC and small molecules were analysed, in addition to processing the subsequent data, by Helen Martin and Aditya Malkar, respectively. All elemental data analysis and all data harvesting were done by the author.

3.1 Removing elemental impurities from skin patches to be used for skin sampling during physical stress

Due to high elemental concentrations observed in unconditioned patches in Chapter 2, this experiment sort to remove as many of these impurities as possible using a new chemical cleaning technique. 3M™ Tegaderm™ +Pad film dressing with non-adherent pads were the chosen patches used for sweat collection. These non-adherent pads are generally used as wound dressings due to a number of properties including a sterile dressing with a waterproof bacterial barrier consisting of non-adherent absorbent pad bonded to a larger thin film dressing.

The objective of this experiment was to reduce elemental impurities in the patches to be used to collect sample sweat. Additionally, once cleaned, patches should be safe to be placed on human skin and not be too dry as to break up upon application onto the skin. H_0: there was no detectable difference in element content of patches following elemental purification by solvent washing i.e. H_0: $Patch_{Blank} = Patch_{Washed}$.

3.1.1 Experimental Outline

8 skin patches were used in this experiment. 7 of these patches were subjected to a series of washes in an attempt to reduce elemental impurities in the patches. Following each wash, 1 patch was removed and stored until all seven washes had been carried out. Once the wash cycle had been completed, elements were leached from the patches in 2% HNO_3 followed by ICP-MS analysis. Elemental concentrations in all patches were calculated and the amount of element reduction was calculated.

3.1.1.1 Wash tray preparation

Two plastic trays (750mm (l) x 600mm (w) x 750mm (h)) were cleaned with several washes of DI H_2O, followed by a wash of aqua regia and a final wash of the proceeding wash solvent. The trays were dried under an infra-red heat bulb (Infrasatin, 240-250 V) overnight.

3.1.1.2 Patch preparation

8 patches were removed from their sterile packets and covers on the back were removed to expose the adhesive backings of the patches. The cotton pads were extracted from the adhesive backings using tweezers.

3.1.1.3 Blank Patch

One of the 8 patches was placed into a 15 ml centrifuge tube. This patch was labelled *Patch 0* and not subjected to any washes. Elemental concentrations from this patch were used as a reference to calculate the reduction of elemental impurities following each wash.

3.1.1.4 Patch washes

7 patches were placed in a plastic tray containing 250 ml of 15% HNO_3 for 6 hours to remove impurities with basic properties, such as alkali's that react with water forming X-OH e.g. LiOH, NaOH, KOH), that may have been present in the patches. The wash solvent was appropriately disposed of and patches were dried in the tray under IR overnight. One patch (*Patch 1)* was removed and placed into a fresh 15 ml centrifuge tube. The remaining 6 patches were placed in a plastic tray containing 250 ml DI H_2O for 6 hours. The DI H_2O was disposed and patches were dried in the tray under IR overnight after which one patch (*Patch 2*) was removed and placed in a 15 ml centrifuge tube. This exact procedure was repeated using different washes (in order): 10% TMAH (*Patch 3*) to remove any acid-soluble impurities i.e. oxides formed with elements with low electron affinity e.g. SiO_2, SO_2, SO_3 etc.; DI H_2O (*Patch 4*); 0.1M EDTA (*Patch 5*) to remove any metal impurities by the chelating effect, where an octahedral geometry is formed with the EDTA forming six bonds to the metal; DI H_2O (*Patch 6*); DI H_2O (*Patch 7*). DI H_2O was used to neutralised the patches so there would not pose any risk to participant skin. Following each wash, one patch was removed and placed in different 15 ml centrifuge tubes (Figure 3.1). This wash order was chosen in order to remove impurities from the left to the right of the periodic table in which the direction of basicity decreases for elements (lower electron affinity).

During the time in which one tray was being used to wash the patches, the other tray was cleaned with several washes of DI H_2O followed by a final wash of aqua regia. The tray was dried under IR overnight and the next wash solvent to be used was prepared and inserted into the tray.

3.1.1.5 Sample Analysis

Following the removal of each patch from the wash cycle, 14 ml of 2% HNO_3 was added to the centrifuge tubes containing the samples patches (supplementary information Table

SI.3.3), in order to leach elemental impurities from the patches into the acid solution. The tubes containing the patches were subjected to 60 minutes of mixing on a roller shaker (ELMI Ltd, Rigia, Latvia) at 35 rpm. Patches were immediately removed from the centrifuge tubes using pre-cleaned (in 2% HNO_3) plastic tweezers to ensure minimum contamination. The remaining solution in the tubes were then analysed by ICP-MS.

Elemental detection of twenty four elements was performed using ICP-MS instrument. Instrument conditions can be found in (Chapter 1 section 1.3.5).

3.1.2 Results and Discussion

A total of eight patch samples were analysed by ICP-MS. From the twenty four elements analysed, ten element impurities could be detected in the untreated blank patch (patch 0). However, only nine of these ten could be quantified (Figure 3.2). Fe could not be quantified but could be detected in only the blank patch (Patch 0). Fe could not be detected in subsequent patches following this first wash with 15% HNO_3. A very high concentration of Na was found in patch 0 at approximately 9000 $ng.mL^{-1}$, just over twenty five times larger than the next largest impurity found, I, which was found at about 350 $ng.mL^{-1}$. High impurities of S and Zn were also observed at approximately 220 and 170 $ng.mL^{-1}$, respectively. Other element impurities observed included Si, Al, K, Ca and P at approximate concentrations of 90, 65, 60, 20 and 10 $ng.mL^{-1}$, respectively (Table 3.1).

All impurities that could be quantified in patch 0 displayed a general decrease in concentration following each successive wash (Figure 3.2). However, following the first wash in 15% HNO_3 the concentrations of I and Si in the patch doubled whereas all other elemental impurities decreased, especially Na. Increasing I and Si concentrations in the patches may be due to the impurities present in the 15% HNO_3 wash solution. Subsequent to this wash the concentration of Na decreased by just over 70%. As a result of this 15% HNO_3 wash, any contamination of P could not be detected and concentration of K and Zn could be detected but not quantified. Concentrations of Al and S also significantly decreased following this wash, by approximately 75 and 80%, respectively. Only the concentration of Ca remained unchanged after this first wash.

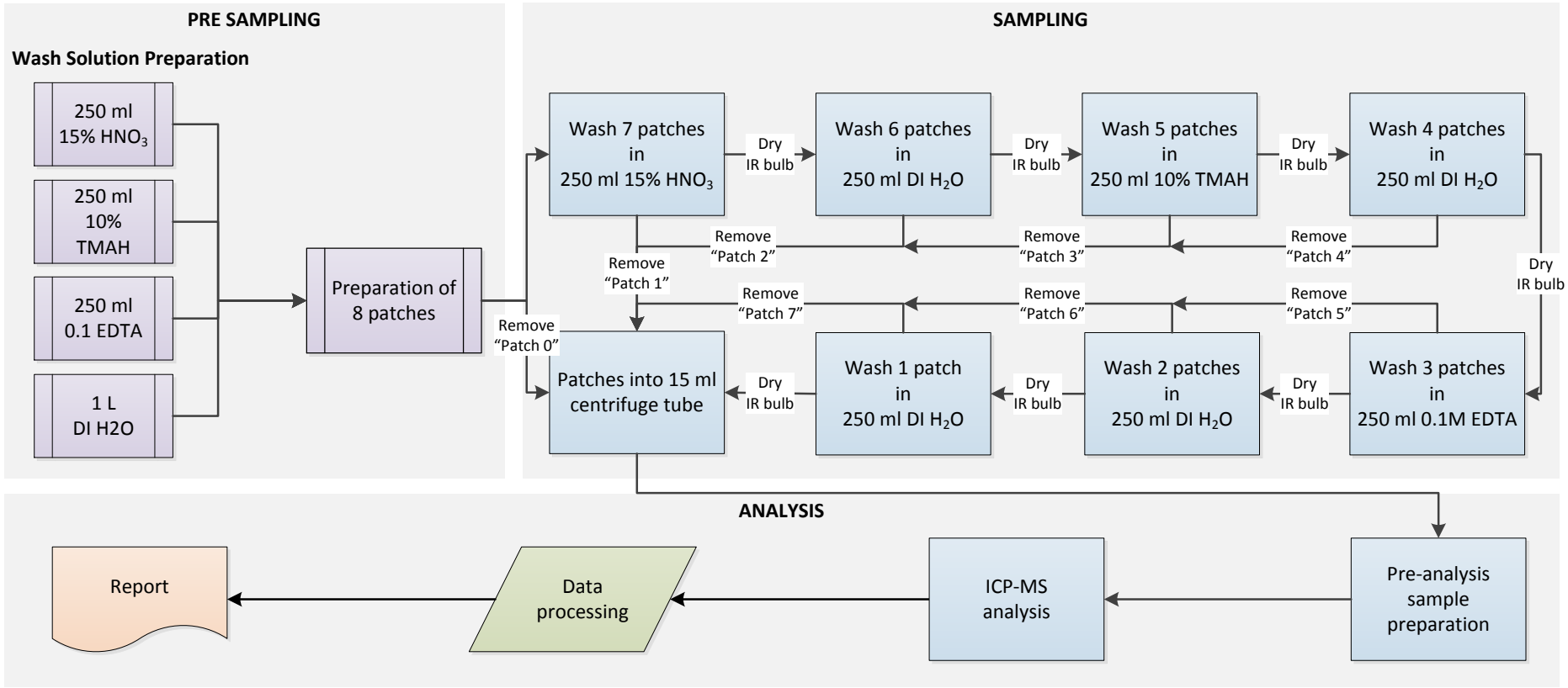


Figure 3.1. Experiment outline for skin patch blanking

Following the second wash in DI H_2O, concentrations of Al, S, Ca and I could no longer be detected. K was still detected but the concentrations were so low that they could not be quantified. Na was still observed at a concentration of 142 $ng.mL^{-1}$ as well as 44 $ng.mL^{-1}$ for S. After the third wash in 10% TMAH only Na and K concentrations could detected. However, only Na could be quantified at 72 $ng.mL^{-1}$. The concentration of Na decreased in the patches subsequent to each wash, until the final wash, in DI H_2O, where a concentration of 17 $ng.mL^{-1}$ was observed.

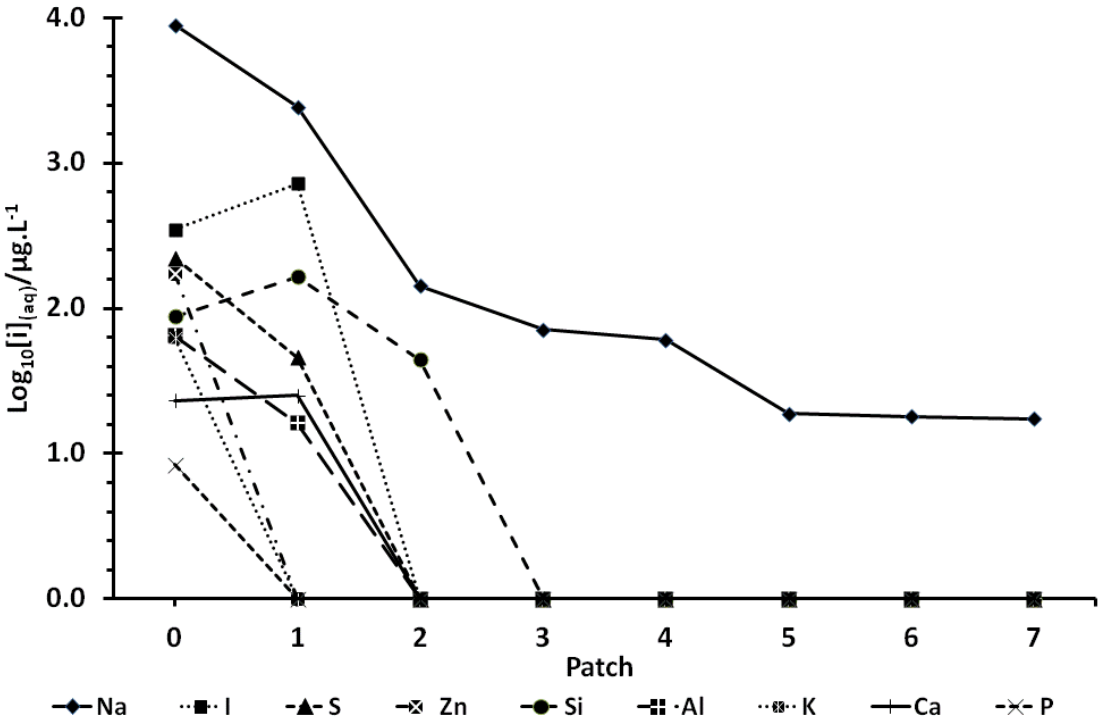


Figure 3.2. Concentration of the nine detected elements, by ICP-MS, in skin patches following each patch wash

3.1.3 Conclusion

H_0 of the experiment was rejected and the alternative hypothesis, there was a detectable difference in element content of patches following blanking by solvent washing, was accepted, i.e. H_0: $Patch_{Blank} \neq Patch_{Washed}$.

All detected elemental impurities in the blank patch were removed from the patches after the first three washes, except Na, where concentration levels in the patches was reduced to an acceptable level of approximately 20 $ng.mL^{-1}$ following wash 5 (Patch 5). After a complete wash cycle the amount of Na in the patches had been reduced by almost 100% when compared to Patch 0.

Table 3.1. Concentrations (ng.mL^{-1}) of elements in patches from washes by SF-ICP-MS, instrument standard deviation (n=5) shown in brackets. Relative change (%) also shown

Patch	Na		Al		Si		P		S		K		Ca		Zn		I	
	[*i*]	R_C (%)	[*i*]	R_C (%)	[*i*]	R_C (%)	[*i*]	R_C (%)	[*i*]	R_C (%)	[*i*]	R_C (%)	[*i*]	R_C (%)	[*i*]	R_C (%)	[*i*]	R_C (%)
0	8916 (62)		65 (0)		88 (0)		8 (0)		222 (0)		63 (0)		23 (0)		173 (0)		349 (0)	
1	2440 (63)	-72.6	16 (15)	-75.1	165 (79)	88.7	< LOD	-100.0	46 (37)	-79.4	**< LOQ**	-100.0	25 (23)	8.9	**< LOQ**	-100.0	719 (0)	106.2
2	142 (3.8)	-94.2	< LOD	-100.0	44 (4.6)	-73.1	< LOD		< LOD	-100.0	**< LOQ**		< LOD	-100.0	< LOD		< LOD	-100.0
3	72 (0)	-49.6	< LOD		< LOD	-100.0	< LOD		< LOD		**< LOQ**		< LOD		< LOD		< LOD	
4	61 (0)	-15.0	< LOD		< LOD		< LOD		< LOD		< LOD		< LOD		< LOD		< LOD	
5	19 (0)	-69.0	< LOD		< LOD		< LOD		< LOD		< LOD		< LOD		< LOD		< LOD	
6	18 (0)	-4.9	< LOD		< LOD		< LOD		< LOD		< LOD		< LOD		< LOD		< LOD	
7	17 (0)	-3.6	< LOD		< LOD		< LOD		< LOD		< LOD		< LOD		< LOD		< LOD	
Change, P0 to P7 (%)	**99.8**		**100**		**100**		**100**		**100**		**100**		**100**		**100**		**100**	

3.2 Analysis of biological fluids from physical stress

Ten male volunteers aged between 18 and 32 years participated in this study. Participants refrained from using soap, shampoo, deodorant and fragrances the night before and the morning prior to the start of sample collection. Participants were also asked to record their dietary intake two days prior to sample collection and were asked not to go swimming nor consume any alcohol during this period. Samples were collected over five consecutive days with participants required for one visit. Two participants were sampled on each day with the first participant arriving at 0745 hrs. and the second at 1015 hrs. Sample collection typically lasted two hours fifteen minutes. The exclusion criteria for this study included participants with any pre-existing conditions, such as asthma, any smokers, participants with any communicable diseases such as a cold or flu, females, participants outside the age range of 18 – 35 years old or carrying any injuries that could be aggravated by cycling on a bike for thirty minutes. The age restrictions for this experiment were put in place in order to attempt to normalise the health of the participant. The male human body within the given age range was likely to be in its optimum condition.

3.2.1 Experimental Outline

Participants were invited to participate in a single three hour session in which samples of breath, blood (plasma), saliva, sweat (skin) and urine were obtained throughout the study as well as the participant's heart rate.

Once changed into shorts, height and weight of participants were measured outside the chamber. A bicycle ergometer (Lode, Groningen, Netherlands) resistance was set at 2 $W.Kg^{-1}$ of total participant body weight. In phase A of the experiment approximately 100 ml of water (in a plastic cup) was consumed before producing a urine sample in order to normalise fluid intake for participants. Two skin patches (one for elemental and the other for VOC analysis) were then attached to the participant's forehead for thirty minutes. A heart rate monitor (Polar CS100, Warwick, UK) was attached around the chest and readings were taken at pre-determined intervals throughout the experiment. A Sensewear^{TM} WMS armband was strapped around the right bicep to gauge the galvanic skin response, measuring the electrical resistance of the skin which is related to moisture levels at the surface. Drool saliva, breath (VOC) and blood samples (right volar) were collected before the skin patches were removed from the forehead.

Phase B (exercise) consisted of the participant cycling at the pre-determined resistance on the bicycle ergometer for thirty minutes. Prior to commencing exercise, a second set of skin

patches were placed on the participant's forehead. During exercise, breath samples (BioVOC) were also collected for differential mobility spectrometry (DMS) analysis. As soon as thirty minutes elapsed, the skin samples were removed and placed into pre-cleaned vessels.

At the end of phase B the participant was escorted back to the sampling area where the third set of skin patches was placed onto the forehead to begin phase C. Saliva, breath and blood samples were collected. 100 ml of water was given to the participant to swallow before producing a urine sample. Patches were removed from the forehead after thirty minutes. Phase D was an exact repeat of phase C. At the end of phase D all attached devices were removed (Figure 3.3).

3.2.1.1 Vials preparation

Blood (plasma): For each participant, sixty 15 ml tubes coated in lithium heparin were obtained from the School of Sport, Exercise and Health Sciences (SSEHS), two every time blood samples were taken. Five 2 ml LoBind cryogenic tubes (Eppendorf, Stevenage, UK) were labelled for each plasma sample resulting in the preparation of 150 cryogenic vials to aliquot plasma. Also, ten 2 ml vials were needed for field blanks (one per participant) and ten for diluting solvent blanks resulting in a total of 170 cryogenic vials prepared for plasma storage.

Saliva: Ten 30 ml glass vials (Chromacol Ltd, Herts, UK) were prepared for saliva collection. Three 2 ml LoBind cryogenic tubes (Eppendorf, Stevenage, UK) were labelled for each saliva sample leading to the preparation of 30 cryogenic vials to aliquot saliva. A further twenty 2 ml vials were prepared for collecting field blanks during sample collection for each participant and also vial blanks. As a result, 50 cryogenic aliquot vials were prepared for saliva storage.

Skin: Sixty patches were prepared using the exact method outlined in section 3.1. For each subject five patches were used, four for sweat collection from the forehead and one for blank patch analysis. The other ten patches were used to collect field blanks, one per participant. Once solvent washed, individual patches were placed in 50 ml screw capped polypropylene tubes. Prior to patch insertion these tubes were pre cleaned, with 2% HNO_3, and air dried.

Urine: A total of thirty 500 ml polyethylene terephthalate (PET) urine containers were labelled, one for each urine sample collected. One hundred and fifty 2 ml LoBind cryogenic

tubes (Eppendorf, Stevenage, UK) were labelled for storage of aliquots following urine collection.

The order in which sample were collected can be observed in Figure 3.4. Physical, physiological, psychological and biological measurements were collected.

3.2.1.2 Physical Stress

Physical stress was induced using a *Corival* electro-magnetically braked bicycle ergometer. Participants exercised continuously for 30 minutes, during the stress phase (Phase B) at a workload of 2 W/Kg of their body weight. Electro-magnetic braking ensured participants always kept working their feet on the pedals. Participants found it more difficult with a low rpm as the electro-magnetic braking would have higher resistance. Consequently, participants tried to keep a consistent rpm where a rhythm was found.

3.2.1.3 Weight & Height

Body assessment was carried out prior to sample collection. Before entering the environment chamber, and whilst wearing only shorts and trainers, participants' weight and height were measured to determine the resistance of the bicycle ergometer and also BMI.

3.2.1.4 Physiological Measurements

A polar heart rate belt was fitted around the participant's chest upon arrival. This remained in place for the duration of the experiment, when it was removed. Heart rate readings were visualised using a watch that was paired to the monitor on the strap. Phase A readings, known as the baseline readings, were logged every ten minutes and included the start reading. Physical stress readings were recorded every five minutes during phase B. Phase B commenced when the participant started exercising and finished when thirty minutes of exercise had elapsed. These two readings were also noted. Measurements were taken every ten minutes during the two recovery phases 1 (phase C) and 2 (phase D). Also of note was the heart rate reading at the end of experiment, at the end of recovery phase 2. All phases were intended to last thirty minutes resulting in two hours of sample and measurement collection.

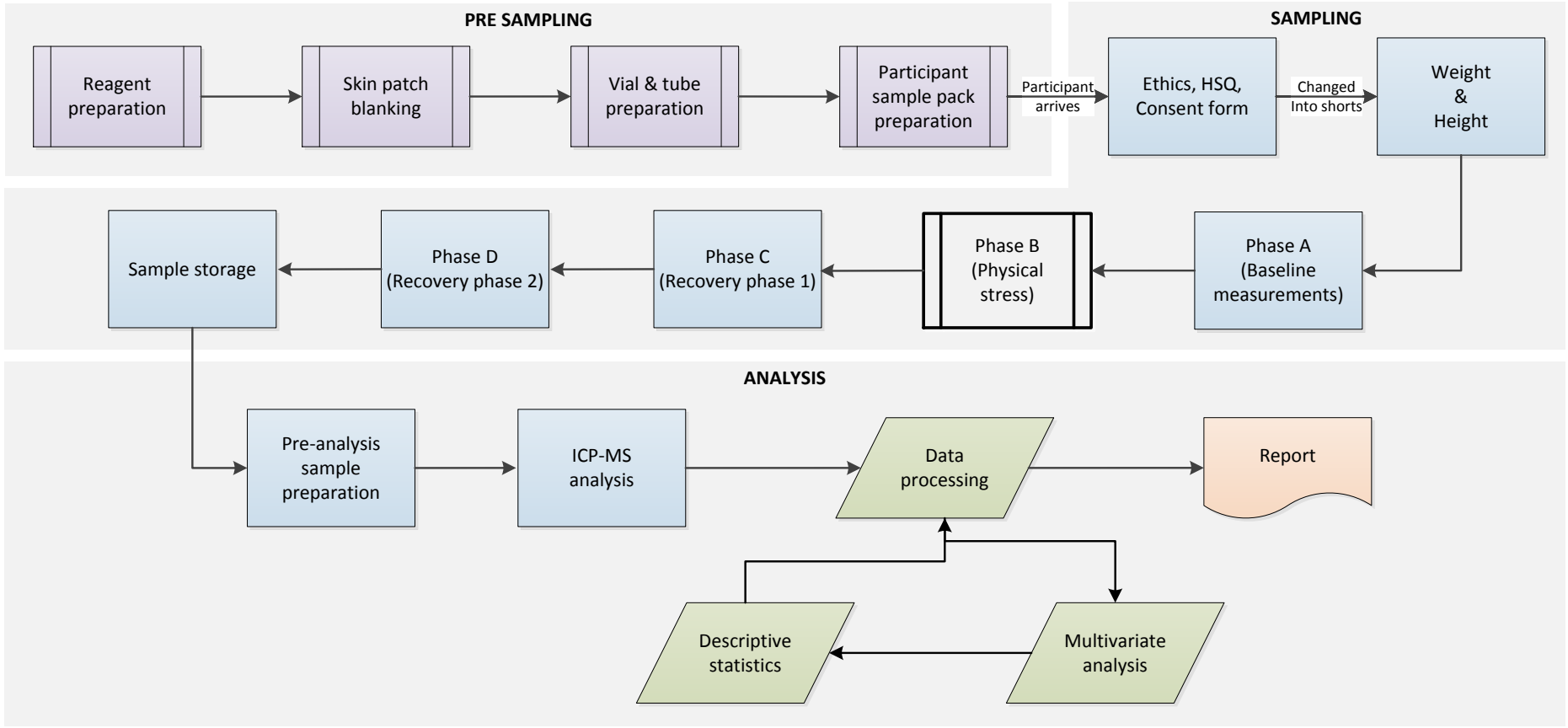


Figure 3.3. Study outline for sample collection and analysis by ICP-MS in physical stress study

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	Skin patches ON (1)	Heart rate monitor ON	Rinse forehead and place skin patches ON (PDMS & cotton)	Skin conductivity meter ON (automatic time stamp)	Saliva (hormones & metals) 1	Saliva (volatiles) 1	Breath 1 (Using breath mask)	Blood 1	Skin patches OFF (1)	Skin conductivity meter manual time stamp 1	Explaination of sampling during exercise	Skin patches ON (2)	Help subject onto bicycle ergometer	Skin conductivity meter manual time stamp 2	10 x DMS readings every 3 minutes (at 2, 5, 8, 11, 14, 17, 20, 23, 26, 29 minutes into exercise)	6 x heart rate readings every 5 minutes (0, 5, 10, 15, 20, 25, 30 minutes in exercise). Please note 0 and 30 minutes are the start and end of the exercise, respectively	Skin patches OFF (2)	Help subject off bicycle ergometer	Skin patches ON (3)	Saliva (hormones & metals) 2	Saliva (volatiles) 2	Breath 2 (Using breath mask)	Blood 2	Allow subject to drink water (100 ml)	Urine 2	Skin patches OFF (3)	Skin patches ON (4)	Saliva (hormones & metals) 3	Saliva (volatiles) 3	Breath 3 (using breath mask)	Blood 3	Allow subject to drink water (100 ml)	Urine 3	Skin patches OFF (4)	Skin conductivity meter off	Heart rate monitor OFF
Questions	Allow subject to drink water/rinse mouth (100ml)																																
Consent form																																	
Information complience																																	
Health Screen Questionnaire																																	

Heart rate readings, time (mins); Relax Phase A: 0, 10, 20, 30; Exercise: 35, 40, 45, 50, 55, 60; Relax Phase B: 70, 80, 90; Relax Phase C: 110, 110, 120

Figure 3.4. Physical stress sample collection timeline

GSR measurements were also recorded for each participant, using the same device described in Chapter 2 section 2.1.1. The Sensewear™ WMS armband. Continuous GSR measurements were obtained from when the armband was placed on the left bicep of participants, activating the device, to when the armband was removed at the end of the experiment, deactivating the device. Four notable time stamps were taken for each participant, the automatic time stamps when activating and deactivating the device, at the start and end of the experiment, and also two manual time stamps (by pressing the *Time Stamp* button on the device) at the end of Phase A, when skin patches were removed, and at the beginning of Phase B, when cycling started.

3.2.1.5 Psychological Measurements

Rating of Perceived Exertion (RPE) was used to determine exercise intensity levels. Perceived exertion is how hard the participant felt their body was working based on physical sensations experienced during physical activity, including increased heart rate, increased sweating, increased respiration or breathing rate, and muscle fatigue. Although a subjective measure, the RPE rating may provide a good estimate of actual heart rate during physical exercise. Borg found a high correlation between a person's RPE and their actual heart rate during physical activity.[88] The rating scale ranges from 6 to 20, where 6 proposes "no exertion at all" and 20 suggest "maximal exertion." Generally, practitioners agree that an RPE rating between 12 and 14 on the Borg Scale suggests physical activity is being performed at a moderate level of intensity. THE RPE scale was used ten times during the 30 minute physical stress phase (Phase B) as participants were asked to rate the RPE every 3 minutes.

3.2.1.6 Biological Sample Collection

Urine, skin patch samples, saliva and blood were collected from each participant at pre-determined stage throughout the study (Figure 3.4).

Urine: Participants provided a total of three urine samples in the experiment during the baseline and recovery phases. During phase A, urine was collected immediately after the height and weight measurements were taken and before the Phase A skin patches were applied to the participant forehead. During phases C and D urine was collected following the collection of all other samples. During these two recovery phases, urine was collected into a 500 ml polyethylene terephthalate (PET) container while skin patches were in place on the participant forehead. The container was then screw capped and handed back to the sample handler who would aliquot 1 ml of urine into labelled 2 ml vials. Aliquots were immediately

placed onto dry ice at -20°C. Prior to collecting each urine sample, participants were asked to consume 100 ml in order to regulate fluid intake and normalise creatinine clearance.

Skin Patches: Four forehead skin patch samples were collected from each participant for thirty minutes for the duration of each phase. Prior to sampling the phase A patch, the participant forehead was wiped with DI H_2O using a pre-cleaned cotton pad, ensuring any possible environmental impurities on the forehead were removed. Cleaned patches were removed from 50 ml centrifuge tubes using polypropylene tweezers and applied to the forehead. The patches were held using pre cleaned cotton pads attached with surgical tape to the forehead. Once thirty minutes had elapsed, the tape and cotton pads were carefully removed and the sample patch was extracted from the forehead using tweezers and placed directly into the 50 ml storage centrifuge tube. Field blanks were also collected for each participant. Upon participant arrival, the tube containing the field blank patch was unscrewed and left in the environmental chambers, until collection of samples was complete and participant left the chamber. At this point the top of the tube was screwed back on. One un-sampled, pre-blanked, patch blank was also collected for each participant.

Following the end of sample collection for each day, all patch tubes were transported back to the Department of Chemistry where 15 ml of 2% HNO_3 was added to each tube containing the sampled skin patch to leach elements from patches. These were left on a roller mixer at a tilt of 5° for 60 minutes. Patches were removed using pre-cleaned tweezers and disposed of appropriately. The tubes containing the sampled solutions were then stored at room temperature until the day of analysis.

Saliva: Three drool saliva samples were collected following the application of forehead skin patches. These samples were collected in 30 ml glass vials during the baseline and recovery phases (A, C and D). Participants were asked to rinse their mouth out, 10 minutes prior to sample collection, to clean the mouth and to stimulate saliva production. Five minutes of time was allocated for each sample of saliva in which participants were asked to place the head forward (looking at the floor), allowing saliva to pool at the front of their mouth and when ready to drool (not to force) saliva into the provided sample tube. The 20 ml collection tube was then screw capped and handed to the sample handler who would aliquot 500 µl of saliva into labelled 2 ml vials. Aliquots were immediately placed onto dry ice at -20°C.

Blood (plasma): At three pre-determined sample points a total of six whole blood samples were taken from the left volar (AE) of each participant. The left arm was used to collect blood due to the collection of GSR measurements from the upper right arm. Blood was

collected, following saliva collection and prior to removal of the skin patch samples in the baseline and recovery phases (A, C and D), using a butterfly cannula and 15 ml collection tubes coated in lithium heparin. For each sample point the cannula was inserted into the volar (AE) and two tubes of 10 ml blood were collected (20 ml in total for each sample point). Collected whole blood was handed to the sample handler who spun the blood down to approximately 50%, using a refrigerated centrifuge for 15 minutes at 2,000 x g, in order to extract the plasma. With the vial placed in ice between 2-8°C, the top layer, containing only the blood plasma, was then extracted using a pipette and separated into aliquots of 500 µl into labelled 2 ml tubes. Aliquots were immediately placed onto dry ice at -20°C.

3.2.1.7 Sample Storage

Following collection and aliquot separation, urine, saliva and plasma were initially stored on dry ice at -20°C. Concluding sample collection for each participant, samples were stored in purpose constructed polystyrene storage containers and placed in -80°C freezers.

3.2.1.8 Sample Preparation

Saliva, urine and plasma samples were stored at -80°C until the day of analysis. Samples were allowed to thaw at room temperature for 1 hour after which all samples were homogenised using a vortex mixer. Skin patches were prepared for analysis on day of sample collection.

Skin patches: Pre analysis preparations for skin patches are outlined in section 3.2.1.6. Prior to elemental analysis, 50 $ng.mL^{-1}$ In was added to all samples as an internal standard (supplementary information Table SI.3.3).

Saliva: Samples were stored between 144 and 164 days. Following homogenisation, all samples were diluted in 2% HNO_3 and 60 $ng.mL^{-1}$ In was added as an internal standard to give a dilution factor of approximately 1:20 (saliva to total volume), in 15 ml polypropylene centrifuge tubes (supplementary information Table SI.3.5).

Urine: Urine samples were stored between 229 and 274 days before analysis. Once thawed and homogenised on day of analysis, samples were diluted fivefold with 2% HNO_3 and 50 $ng.mL^{-1}$ Ru was added as an internal standard in 15 ml polypropylene centrifuge tubes (supplementary information Table SI.3.7).

Plasma: Storage of plasma samples lasted between 525 and 529 days. Once homogenised on the day of analysis, plasma samples were diluted fifty fold using 2% HNO_3 in 15 ml polypropylene centrifuge tubes (supplementary information Table SI.3.9). An external

standard, to monitor the stability of the instrument, containing 5 ng.mL^{-1} MES1, MES2 and MES4 (Spex Certi Prep) was prepared and analysed between each sample during an analysis sequence on the ICP-MS.

3.2.1.9 ICP-MS Analysis

Elemental analysis of the four biological samples above was performed using ICP-MS. Instrument conditions can be found in Chapter 1 section 1.3.5.

3.3 Results and Discussion

In addition to the physiological measurements, data acquired from biological sample measurements are also presented in this section. Using the heart rate data, which was obtained throughout the experimental phases for all participants, alongside body mass index and rate of perceived exertion data, a newly formulated equation was constructed to measure participant physical stress scores. MVA and descriptive statistics was performed using the data obtained from ICP-MS data that included the analysis of twenty four elements in sixty skin patch samples, twenty seven elements in twenty seven saliva samples, twenty seven elements in thirty urine samples and thirty five elements in twenty four plasma samples. Using common observations and variables between the elements and participants from the four biological sample types, the exploration of patterns in data was analysed using MVA. Further to this, common observations and variables in elemental (ICP-MS), VOC (TD-GC-MS) and small molecules (UPLC-IM-MS) data were also analysed using five participants.

3.3.1 Physiological measurements

Individual heart rate was collected throughout the experimental period and the average observations show the relative change in cardiovascular responses during the stress phase. Participant heart rates remained stable for the initial thirty minutes of the study, while baseline measurements were obtained. During the stress phase (phase B) participant heart rate significantly increased. Initially, this considerable increase was the consequence to urgently distribute oxygen around the body following the work required for exercise. Towards the end of phase B the heart rate only increased gradually as the work forced on the heart reached its maximum.

Paired t-tests were carried out to find if any significant difference in heart rate between the four phases existed. H_0: There was no difference in heart rate between the non-exercise and exercise phases (Appendix Table 8.8). Evidence of significant differences (H_0 rejected) in participant heart rates were present for all ten participants between phases A and B, nine

participants between phases B and C and only three participants between phases C and D. This statistical evidence along with the heart readings confirms that participant heart rate did increase between rest and exercise, during phases A and B, respectively. Recovery then began in the following phase (phase C) for all participants except participant CZ9 whose heart rate was still very high and thus recovery was slower. Between phases C and D only participants CZ3, CZ8 and CZ9 had heart rate readings that had not stabilised whilst all other participant heart rates had stabilised. Interestingly, nine out of ten participant heart rates were found to be significantly different between phases A and D. Observing the heart rate data shows that whilst heart rates for the majority of participants had stabilised, the readings were higher following stress compared to prior to the intervention during phase B.

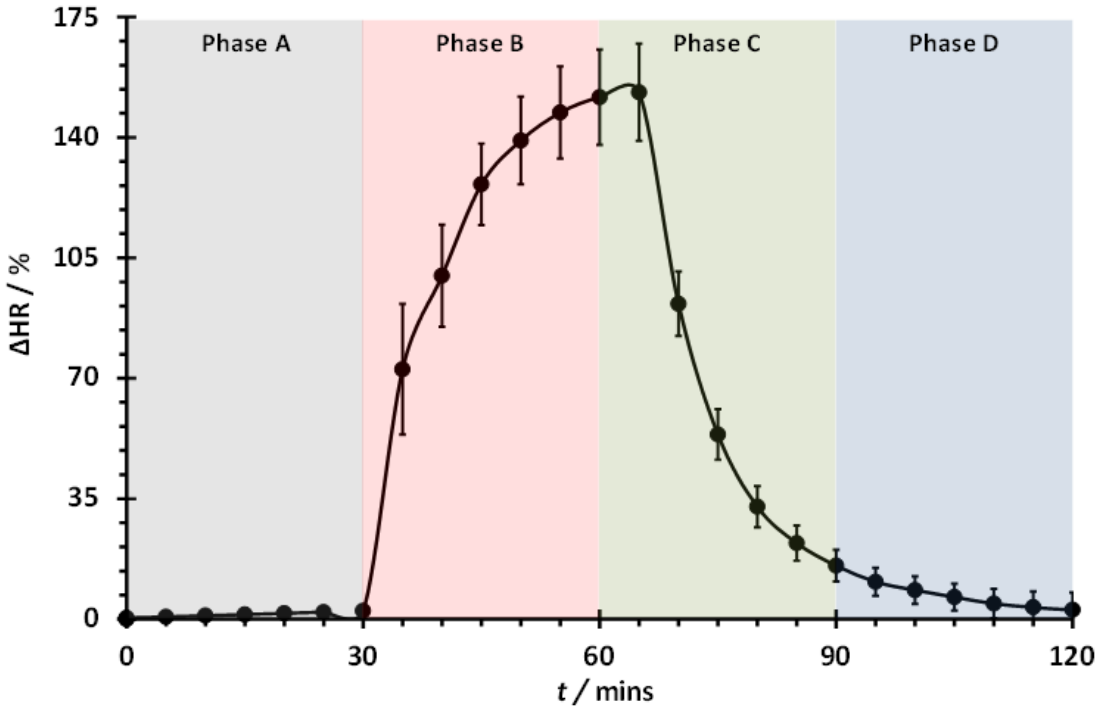


Figure 3.5. Mean relative change for heart rate responses (ΔHR) of 10 male participants over the 120 minute session. The error bars designate 95% confidence limit on the mean

3.3.1.1 Physical Stress Response Scores

Participants' individual cardiovascular response to the stress intervention was scored on the basis of observed changes in their heart rate and BMI. The equation was adapted from the physical stress response score (Equation 2.1) created by Matthew Turner.[87] The relative change (ΔHR (%)) of the individual's heart rate was calculated by first subtracting the maximum heart observed during phase B (HR_{MAX}) from the baseline heart rate (HR_0), which was calculated by averaging readings taken during phase A, and then dividing the resulting value by HR_{MAX}. This number was multiplied by 100. ΔHR (%) was then multiplied by ΔHR. The final operation was multiplying this overall answer by the BMI, which was calculated by

dividing the individual's weight (W), in kilograms, by the height^2 (H^2), in meters^2. Equation 3.1 defines the Physical stress response score (SR_{PHY}).

$$SR_{PHY} = \Delta HR\ (\%)\ X\ \frac{\Delta HR}{HR_{MAX}}\ X\ BMI$$

where

$$\Delta HR\ (\%) = \frac{(HR_{MAX} - HR_0)}{HR_0}\ X\ 100$$

(Equation 3.1)

and

$$\Delta HR = \frac{(HR_{MAX} - HR_0)}{HR_{MAX}}$$

and

$$BMI = \frac{W}{H^2}$$

The SR_{PHY} was devised to emphasize the individuals showing largest cardiovascular response during phase B. The SR_{PHY} scores for the participants are summarised in Table 3.2. The SR_{PHY} score where individuals with a lower score were said to not be stressed was calculated as SR_{PHY} = 1099. This was calculated by using Equation 3.1 and removing the phase B and first two phase C heart rate readings from the individual with the highest SR_{PHY} which was participant CZ9. Therefore participant CZ6 (SR_{PHY} = 917) was not under physical stress during his trial whereas all the other nine participants were said to be under physical stress. It was later discovered that CZ6 was an athlete who trained at least days a week and therefore less susceptible to stress given the workload. The average response for the nine participants was a SR_{PHY} of 2497 ± 1107.

Table 3.2. Physical stress response scores of participants during the exercise phase (SR_{PHY})

Ref.	1	2	3	4	5	6	7	8	9	10
BMI	22.3	24.4	23.1	21.7	23.8	22.4	25.1	27.8	32.1	31.4
RPE_{MAX}	11	18	15	14	15	11	18	19	20	19
HR_0	58	64	75	63	70	61	75	69	82	58
HR_{MAX}	152	173	159	169	188	114	182	176	218	154
ΔHR (%)	164	172	113	169	168	88	142	156	166	167
$\Delta HR/HR_{MAX}$	0.62	0.63	0.53	0.63	0.63	0.47	0.59	0.61	0.62	0.63
SR_{PHY}	2274	2664	1390	2310	2498	917	2089	2654	3323	3272
SR_{PHY}^{N}*	0.68	0.80	0.42	0.70	0.75	0.28	0.63	0.80	1.00	0.98

*SRPHY normalised to highest SRPHY obtained from all participants in the study

Despite a constant workload throughout phase B, participants felt they were working harder as the 30 minutes elapsed and this was reflected in the RPE scores. The average RPE response for all participants was 13.7 ± 6.7. However, for the scores between 0-15 minutes

was 12.6 ± 5.6 whilst those observed between 16-30 minutes were higher with an average RPE score of 14.7 ± 4.7. This data suggests that the participants felt that more physical stress was exerted in the second part of the intervention when compared to the first part. Also, less variability was observed in the second part of the intervention where participants felt a varying degree of stress in the first part.

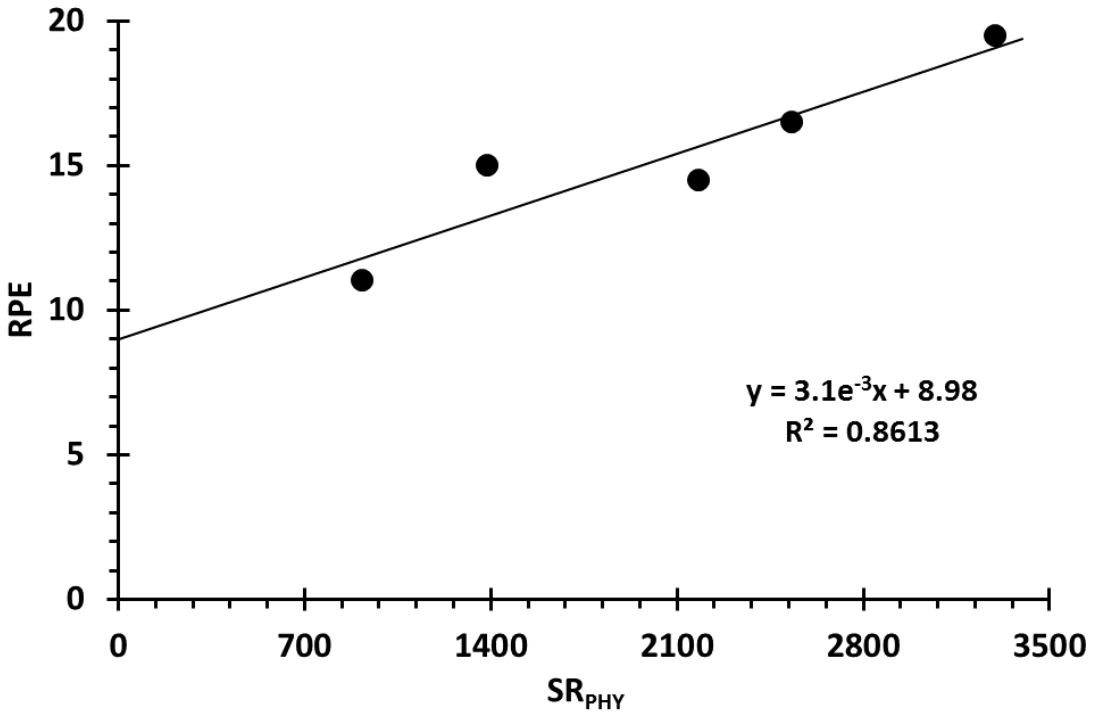


Figure 3.6. The correlation between physical stress response score (SR_{PHY}) and rating of perceived exertion (RPE). Black dots show average of two data points.

Interestingly, when this psychological RPE score is compared against the SR_{PHY} score, a weak positive correlation is identified (Figure 3.6). Generally, participants who gave a higher RPE score were found to have a high SR_{PHY} score indicating they were able to assess their physical state well.

3.3.2 Skin Patches

60 participant skin patches were stored between 73 and 86 days and were analysed over a 13 day period by ICP-MS. 6 analytical sequences were conducted over 3 days (supplementary information Table SI.3.3). For each sample patch, element concentrations obtained from corresponding blank and field blank patches were subtracted. 10 elements were detected in participant skin patches from the 24 elements that were analysed.

It is a possibility that elemental markers discovered from forehead skin may possibly show the effect of physical stress occurring at a different site in the body. For example, it is known that one of the biological pathways for forehead sweat glands is the digestive system

(stomach). Consequently, elemental changes in forehead skin, such as those observed for the elements K, S, Ca, Mg and Na, may in fact point to the differences in elemental concentrations released via the stomach following physical stress.

3.3.2.1 Multivariate Analysis

The skin data set used for MVA consisted of the 10 detected element concentrations (variables) against the participants' skin patches (observations). PLS-DA was initially performed on skin patches obtained from all four phases of the study. The S-plot generated from this statistical model (Figure 3.7) indicated five potential elements from forehead skin that changed in response to induced physical stress experienced by the participants; Na, Mg, S, K and Ca. PCA of these five elements enabled separation between the baseline (phase A) and the physical stress (phase B) observations. This score plot, in addition to the recovery observations, is given in Figure 3.7.

In Figure 3.7 responses from phases A and B appear separated. Phase A observations are clustered relatively close to each other, in two groups, whilst phase B observations can be observed in two clusters, away from phase A observations. Although a distinction can be made for between baseline and stress measurements, the two phase A clusters could indicate two groups of elemental biomarkers between the stress observations themselves. Observations CZ8 and CZ2 are almost adjacent to the two clusters where the stress observations are located suggesting that participant 2 and 8 have an initial slow stress recovery. However, both participant observations for the final recovery phase appear to be clustered with all other final recovery observations indicating that between thirty minutes and sixty minutes following stress their elemental levels found in skin are closer to those observed during phase A (baseline values) and thus all participants have recovered by then. Interestingly, participant 9 shows the slowest recovery and is not fully recovered to baseline levels by the time the experiment is completed. The final recovery observation, CZ9 D in Figure 3.7, shows that the blue observation is grouped within the first recovery observations, which have yet to fully recover following the physical stress. Participants 1, 6 and 10, are the quickest to recover following physical stress due to the grouping of the observations CZ1 C, CZ6 C and CZ10 D, respectively, within the final recovery (phase D) observations. The principal components identified exhibit 86.3% [PC1] and 12.5% [PC2] of the total explained variance of the data set for physical stress yielding a 100% sensitivity (% of correctly assigned true positive observations) for the physical stress observations and 100% selectivity (% of correctly assigned true negative observations) for the baseline measurements.

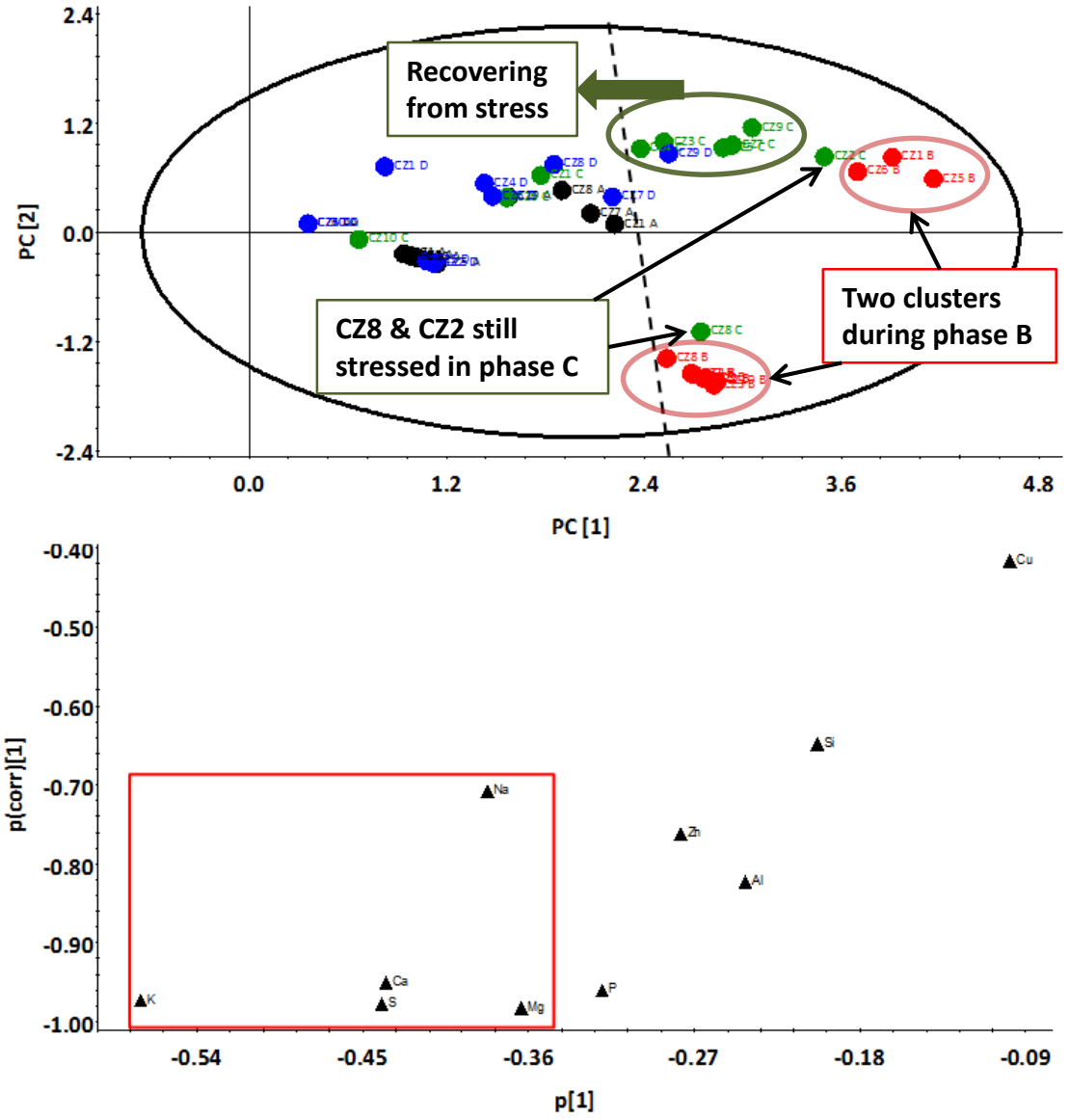


Figure 3.7. Top: PCA scatter plot of five stress sensitive skin components identified from PLS-DA s-plot on 10 observations each from the baseline (black dots), physical stress (red dots), recovery phase 1 (green dots) and recovery phase 2 (blue dots) measurements from ten male participants. 100% sensitivity was obtained for stress phase observations and 100% selectivity was obtained for baseline measurements. These were calculated using the dotted line running through the ellipse obtained from the score plot dendrogram Bottom: S-plot generated from the modelled covariance (P(corr)[1]) and modelled correlation (p[1]) of the PLS-DA from the concentration of 10 skin sensitive elements (variables) from 40 skin samples (observations). The five variables highlighted (red square) were found to provide the highest magnitude (concentration) of variables in the data set and greatest reliability (P(corr)[1]axis) of the variables in the data set, indicating potential elements sensitive to exercise

3.3.2.2 Descriptive statistics

For the detected elemental concentrations, ANOVA (two-factor) was performed for skin patch samples which were grouped into the four phases. A significant difference was observed (95%) between the sample groups with a calculated F value of 14.60 which was much higher than the critical F value of 2.63 (Appendix Table 8.4). Subsequently, a paired t-

test was carried out on normalised data (log_{10}) to determine if skin patch concentrations were statistically different between different phases (Table 3.3). For this test, H_0 implied that no differences in element concentrations in participant skin patches were present between the four phases. From the ten elements detected, H_0 was rejected between phases A (baseline) and B (stress) for the six elements Mg, Al, P, S, K and Ca. The largest relative increase from these six was observed for K (13500%) followed by Mg (5550%) and S (2900%). P increased by 1200% whilst Al and Ca also increased by 400% and 300% respectively. This increase suggests that these elements have been secreted through the pores on the forehead and absorbed into the skin patch.

Table 3.3. Concentration (ng.mL^{-1}), change (%) and paired t-test (P) results of 10 detected elements found from participant skin patches for each phase. Bold values indicate statistical significance at 95%

Phase		Na	Mg	Al	Si	P	S	K	Ca	Cu	Zn
A	μ	45	1	1	7	2	10	84	70	2	101
	σ	61	4	5	12	6	17	123	175	4	273
B	μ	7617	70	8	6	27	303	11479	275	4	37
	σ	13095	28	11	9	21	136	6347	96	2	94
C	μ	3371	7	2	8	6	108	1112	101	1	6
	σ	4365	18	6	13	16	281	1577	223	2	11
D	μ	236	0	3	9	0	7	95	25	0	6
	σ	497	0	6	20	0	11	157	41	0	11
Between phases											
Δ(%)	A & B	16968	5550	431	-13	1252	2937	13606	294	118	-63
	B & C	-56	-90	-77	25	-76	-64	-90	-63	-76	-83
	C & D	-93	-100	67	14	-100	-93	-91	-75	-100	-8
	A & D	428	-100	101	24	-100	-29	14	-64	-100	-94
t test (P)	A & B	0.101	**0.000**	**0.005**	0.812	**0.004**	**0.000**	**0.000**	**0.006**	0.255	0.514
	B & C	0.393	**0.001**	**0.011**	0.717	**0.036**	**0.033**	**0.001**	0.068	**0.018**	0.323
	B & D	0.112	**0.000**	**0.023**	0.719	**0.002**	**0.000**	**0.000**	**0.000**	**0.000**	0.334
	C & D	**0.042**	0.199	0.377	0.914	0.187	0.280	0.071	0.323	0.134	0.895
	A & D	0.246	0.584	0.113	0.841	0.344	0.743	0.787	0.465	0.272	0.301

No statistically significant decreases in elemental concentrations were observed between the phase A and B. H_0 was also rejected between phases B (stress) and C (recovery phase 1) for six elements Mg, Al, P, S, K and Cu. Intriguingly, all six element concentrations immediately decrease following the stress phase. Between the end of phase B and the first recovery phase element concentrations changed for Mg, Al, P, S, K and Cu by -90, -77, -76, -64, -90 and -76 %, respectively, suggesting that recovery was underway immediately following the stress phase. H_0 was accepted for all elements between the two recovery phases (B and C) except for Na, where there was evidence of significant difference. However, between phases A (baseline) and D (recovery phase 2) H_0 was accepted for all elements using this statistical test and therefore there was no statistically significant evidence of a difference. This could also suggest that elemental concentrations in participant

forehead skin patches have returned to baseline levels observed prior to the physical stress intervention.

This is confirmed in Table 3.3 as all detected elemental concentrations between phases A and D are similar when compared to the stress phase, B, where elemental concentrations for the six elements previously mentioned increased.

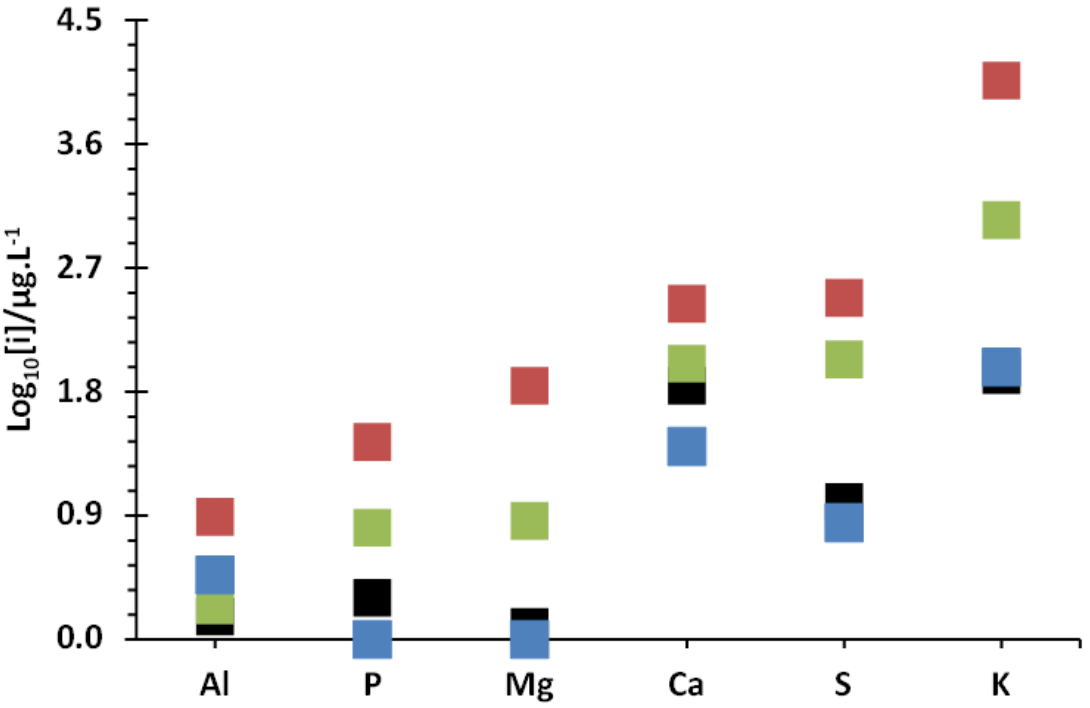


Figure 3.8. Scatter plot showing the average concentration (n = 10) of the six elements where statistical significance was observed for baseline (black), physical stress (red), recovery 1 (green) and recovery 2 (blue) participant skin patch samples

This trend is reiterated in Figure 3.8, which displays the concentrations of the six detected elements. Elemental skin patch concentrations increase to their highest concentration during the stress phase before reducing to somewhere between the concentrations found for the phase A and B, for the first recovery phase, before returning close to the phase A concentrations during the second recovery phase.

3.3.3 Saliva

27 participant drool saliva samples were collected. However, only 7 complete sets of samples were collected. For participants CZ3, CZ7 and CZ10 saliva from only two out of the three sample points were collected due to lack of saliva production as a consequence of dehydration. Consequently, the data for these participants have been excluded from the following statistics. Samples were stored between 144 and 164 days and were analysed over a 20 day period by ICP-MS. 7 analytical sequences were conducted over 16 days

(supplementary information Table SI.3.5). Calibration standards were used to calculate elemental concentrations and corrected using an In internal standard. Element concentrations obtained from the dilution matrix (2% HNO_3) and collection tube blanks were then removed from each participant sample. 8 elements were detected in participant drool saliva from the 27 elements that were analysed (supplementary information Table SI.3.6).

3.3.3.1 Multivariate Analysis

The saliva data set consisted of the concentrations of 8 detected elements (variables) against the participants' drool saliva samples (observations). PLS-DA was initially performed on the saliva data set collected from the three phases of the study. The S-plot generated from this statistical model (Figure 3.9) indicated five potential elements from drool saliva changed in response to physical stress experience by the participants; Na, P, S, K and I. PCA of these five elements provided some separation between baseline (phase A) and recovery 2 (phase D) observations (Figure 3.9). The principal components identified exhibit 99.3% [PC1] and 0.6% [PC2] of the total explained variance of the data set for physical stress yielding a 71.4% sensitive for recovery 1 observations and 71.4% selectivity for the baseline measurements.

Although some separation is observed there are no clear patterns in the data from MVA. However, examining the individual observations for each participant showed varying elemental changes in saliva resulting from physical stress for each participant. Observations A and C for participants 2 and 4 are close together whereas there is a definite change when compared to the corresponding phase D observations. For participants 1 and 5, phase A observations appear to be different to the corresponding phases C and D observations, which are similar. The phase C observation differs from phases A and D observations for participant 6, which could possibly suggest good recovery, whereas there is only a negligible change in phases A, C and D for participants 8 and 9. Possibly, this could suggest that participant 6' elemental saliva composition changes quickly and followed by the quickest recovery whereas the elemental change in saliva for participants 8 and 9 either does not change at all or the change was too slow to observe over the given time period. Somewhere in between these two extremes are participants 1 and 5 whose elemental saliva composition appears to change reasonably quickly but the recovery is slow whereas participants 2 and 4 appear to have a delayed change in elemental saliva composition and an even slower recovery.

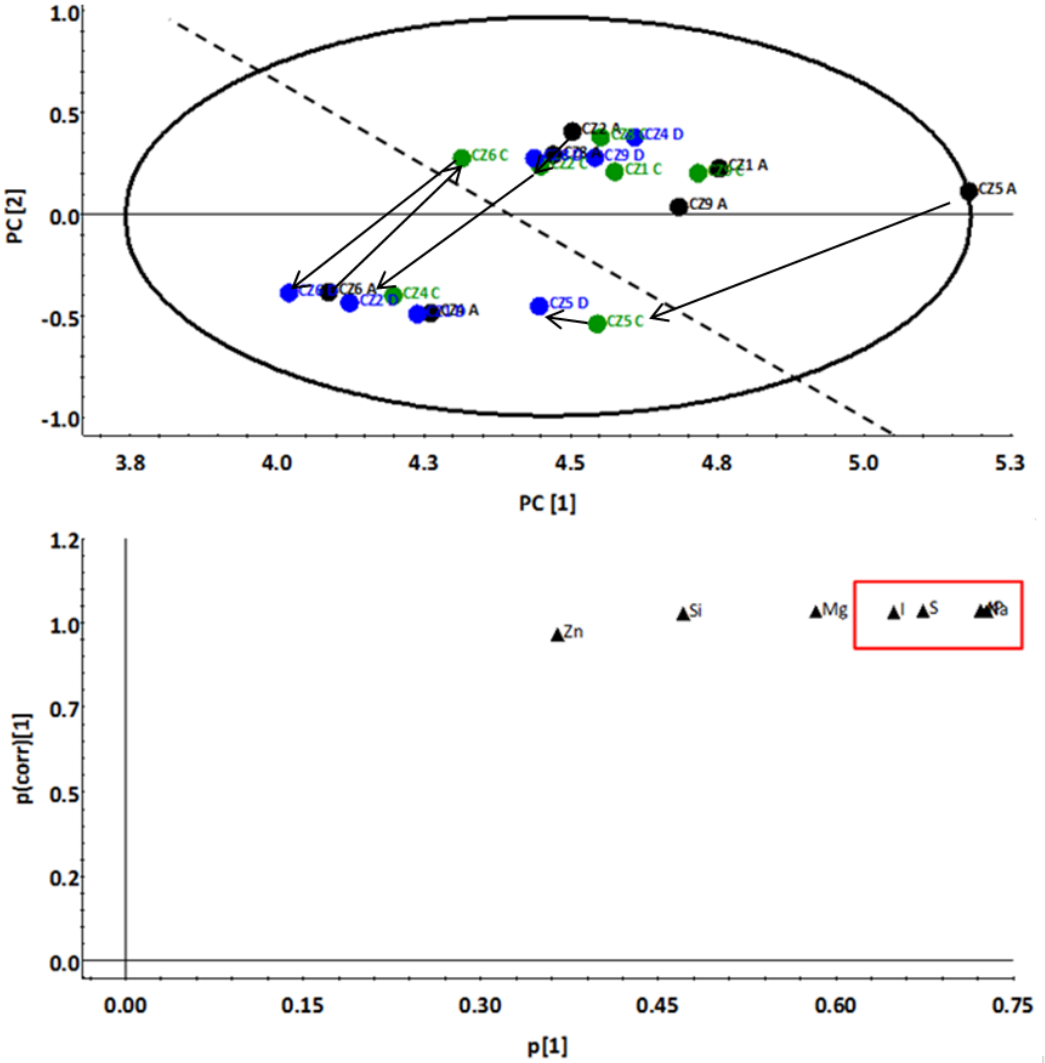


Figure 3.9. Top: PCA scatter plot of five stress sensitive saliva components identified from PLS-DA s-plot on 7 observations each from the baseline (black dots), recovery phase 1 (green dots) and recovery phase 2 (blue dots) measurements from seven male participants. 71.4% sensitivity was obtained immediately following the stress phase and 71.4% selectivity was obtained for baseline measurements. Bottom: S-plot generated from the modelled covariance (P(corr)[1]) and modelled correlation (p[1]) of the PLS-DA from the concentration of 8 saliva sensitive elements (variables) from 27 saliva samples (observations). The five variables highlighted (red square) were found to provide the highest magnitude (concentration) of variables in the data set and greatest reliability (P(corr)[1]axis) of the variables in the data set, indicating potential elements sensitive to exercise

3.3.3.2 Descriptive Statistics

The major elemental contributions in the composition of human saliva are from the presence of K, P and Na (Table 3.4). ANOVA (two-factor) was performed using the elemental concentrations of the saliva collected from three phases. The calculated F value (1.49) was lower than the critical F value (3.06) therefore no statistically significant evidence was observed to suggest the presence of a significant difference (95%) between the groups (Appendix Table 8.5).

Table 3.4. Concentration (ng.mL-1), change (%) and paired t-test (P) results of 8 detected elements found from participant drool saliva collected from three phases. Bold values indicate statistical significance at 95%

	Phase	Na	Mg	Si	P	S	K	Zn	I
A	μ	21635	763	92	25917	6883	63621	25	7216
	σ	13828	622	67	11307	4123	58870	28	9434
C	μ	21945	689	72	22189	5684	42507	19	3628
	σ	11265	298	18	6627	2280	31810	12	3656
D	μ	22024	610	68	20441	4723	31577	21	2678
	σ	7315	235	39	4210	1504	43072	13	2033
Between phases									
Δ (%)	A to C	1	-10	-21	-14	-17	-33	-24	-50
	C to D	0	-12	-6	-8	-17	-26	12	-26
	A to D	2	-20	-26	-21	-31	-50	-15	-63
t test (P)	A to C	0.886	0.834	0.903	0.124	0.177	0.865	0.429	**0.006**
	C to D	0.507	0.251	0.267	0.565	0.079	0.372	0.295	0.066
	A to D	0.461	0.598	0.252	0.128	**0.029**	0.367	0.492	**0.012**

A paired t-test was carried out on normalised saliva data (log_{10}) to find significant evidence of differences in elemental saliva between the phases in which the samples were collected (Table 3.4). H_0: no differences in elements from participant saliva were present between the phases where the samples were collected. H_0 was rejected between phases A and D for S (50% decrease) and between the phases A to C (50% decrease) and A to D (63% decrease) for I. All elements in participant saliva samples decreased in concentration following each phase of the study except for Na, which remained constant, and Zn between phases C and D, which increased between these phases after initially dropping in concentration between phases A and C.

Interestingly, a high but unexpected level of I is observed. It is known that salivary glands secrete iodide in the saliva at a concentration higher than in the plasma. In the majority of the population iodine is introduced into the body via dietary intake. Furthermore, Iodine has an important role to play in oral mucosa and in salivary glands physiology. Salivary glands derived from primitive I-concentrating oral cells, migrate and specialise in secretion of saliva and iodine. Gastro salivary clearance and secretions of iodides are a considerable part of gastro-intestinal cycle of iodides, which constitutes to about 23% of iodides pool in the human body.[89] An unexpected Si concentration is also recorded in the saliva data. It is believed that the presence of Si in the elemental saliva samples is due to the collection method of VOC saliva, which used a polydimethylsiloxane (PDMS) rod, as this rod was inserted into the mouth prior to elemental saliva sample collection.

Elemental changes found in saliva may be an indication of stress affects from around the body as many of the major biological pathways are connected to the salivary glands in the

mouth. However, the data presents that changes in elemental concentrations in salvia, as a result of physical stress, are slower than the timeframe of the experimental time used in this study.

3.3.4 Urine

30 participant urine samples were split into 3 replicates and stored between 229 and 274 days. 91 urine samples were analysed over a 45 day period by ICP-MS. 10 analytical sequences were conducted over 8 days (supplementary information Table 4.7). For each sample, concentrations were determined using calibration curves and a Ru internal standard. Blank elemental concentrations were subtracted from those obtained from the two urine collection pots analysed. 8 elements were detected from the 27 elements analysed (supplementary information Table SI.3.8).

3.3.4.1 Multivariate Analysis

Eight detected element concentrations (variables) against participants' urine samples (observations) were used to construct the urine data set. PLS-DA was originally performed using the concentrations obtained from the three phases of the study after which an s-plot was modelled using this supervised model (Figure 3.10). From the s-plot two potential elemental biomarkers in urine as a result of stress were identified; S and K. PCA of these two elements displayed a separation between the baseline (phase A) and recovery 1 (phase C) observation with two principal components displaying 93.5% [PC1] and 4.44% [PC2] of the total explained variance of the data set for physical stress yielding 80% sensitivity for the recovery 1 measurements and 60% selectivity for the baseline measurements. Figure 3.10 clearly shows a cluster of observations for recovery 2, whilst the baseline observations are scattered and the recovery 1 observations are clustered together. This could suggest that following stress there are different changes in elemental concentrations for urine showing elemental stress markers whilst recovery 2 concentrations all change towards the observations for the initial baseline concentrations. A few recovery 2 observations, back on the same side as the baseline observation are also similarly scattered to show the recovery in the participants' kidneys following physical stress. However, a number of recovery 2 observations are within or close to the recovery 1 cluster suggested that stress recovery in these participants are slow where participants have not fully recovered even one hour after the thirty minute intervention.

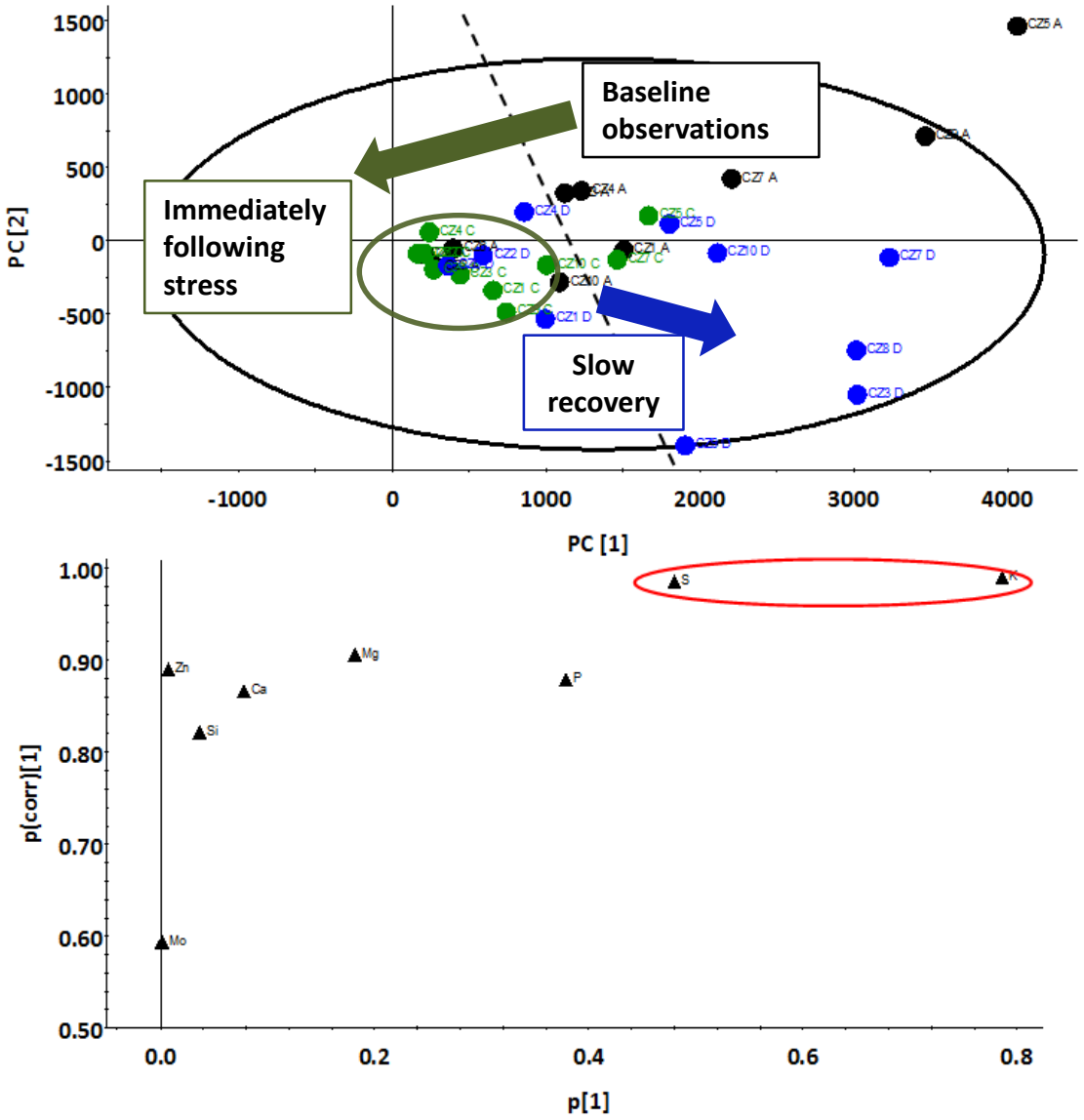


Figure 3.10. Top: PCA scatter plot of two stress sensitive urine components identified from PLS-DA s-plot on 10 observations each from the baseline (black dots), recovery phase 1 (green dots) and recovery phase 2 (blue dots) measurements from ten male participants. 80% sensitivity was obtained immediately following the stress phase and 60% selectivity was obtained for baseline measurements. Bottom: S-plot generated from the modelled covariance (P(corr)[1]) and modelled correlation (p[1]) of the PLS-DA from the concentration of 8 urine sensitive elements (variables) from 40 saliva samples (observations). The two variables highlighted (red square) were found to provide the highest magnitude (concentration) of variables in the data set and greatest reliability (P(corr)[1]axis) of the variables in the data set, indicating potential elements sensitive to exercise

3.3.4.2 Descriptive Statistics

Eight elements were detected in participant urine samples mainly comprising of K, P and S. Other notable contributors included Mg, Ca and Si whilst small amounts of Zn and Mo were also detected. Large changes were observed for all elements detected between all phases with notable changes between phases A and C for P (-72%), Mg (-51%) and Zn (-51%), between phases C and D for Mg (269%), Zn (241%), Si (163%), P (162%) and S (162%), and between phases A and D for Si (97%) and Mg (82%). ANOVA (two-factor) indicated a significant difference (95%) was present between the baseline and recovery measurements

with a calculated F value (6.00) higher than the critical F value (3.04) proving statistically significant differences (Appendix Table 8.6). A paired t-test on normalised data (log_{10}), to find any statistically significant evidence of differences, was carried out where H_0 indicated no elemental differences were present in urine between the three phases (Table 3.5).

H_0 was rejected for seven elements in thirteen of the twenty six tests conducted. Significant differences were present between phases A and C for Mg, P, S, and Ca, between phases C and D for Mg, Si, P, S, K, Ca and Zn, and between phases A and D for Si and Zn. Interestingly, all the element concentrations in urine decrease, from those observed for the baseline readings, following physical stress. This effect is more than likely due to initial dehydration as the participants worked their bodies producing sweat and thus reducing the need to excrete excess water. All elemental concentrations then increase during the recovery phase back to or above the baseline concentrations.

Table 3.5. Concentration ($ng.mL^{-1}$), change (%) and paired t-test (P) results of 8 detected elements found from participant urine collected from three phases. Bold values indicate statistical significance at 95%

Phase		Mg	Si	P	S	K	Ca	Zn	Mo
A	μ	185237	8709	1443731	1346512	3973237	52886	370	65
	σ	194955	7898	1469400	954312	2350893	45259	348	83
C	μ	91227	6523	399375	824114	2996548	32373	183	40
	σ	89387	7061	484237	533712	1830530	27423	218	69
D	μ	336434	17145	1045054	2157910	5526014	59413	622	38
	σ	307050	12934	779039	1331612	2612786	41284	347	38
Between phases									
Δ (%)	A to C	-51	-25	-72	-39	-25	-39	-51	-38
	C to D	269	163	162	162	84	84	241	-6
	A to D	82	97	-28	60	39	12	68	-42
t test (P)	A to C	**0.015**	0.113	**0.005**	**0.018**	0.188	**0.038**	0.343	0.150
	C to D	**0.000**	**0.000**	**0.006**	**0.001**	**0.002**	**0.003**	**0.007**	0.799
	A to D	0.069	**0.004**	0.953	0.085	0.167	0.362	**0.048**	0.440

Urine is secreted in the kidneys, which extract soluble waste by cleaning the bloodstream as well as waters, sugars and other compounds including poisons. Elemental changes discovered from urine following physical stress may indicate build of possible toxic compounds within the body. The elements detected are those that have been excreted via the urine suggesting any increase in elemental concentration following physical stress required any build-up of excess waters, sugars or toxins to be excreted in order to protect the body.

3.3.5 Plasma

Oxygenated blood travels around the body from the left side of the heart to the tissues and then back to the right side of the heart and therefore collected blood had been circulated around the body which may show effects of physical stress.

Plasma was analysed instead of whole blood because it is free of minerals, proteins and nutrients. Plasma is left behind when blood is rid of red cells leaving a yellow colour and its components are majority water and blood. 24 participant plasma samples were stored between 525 and 529 days and were analysed by ICP-MS over a 2 day period with 1 analytical sequence performed on each day (supplementary information Table SI.3.9). Elemental concentrations were calculated using calibration curves and corrected by a 5 $ng.mL^{-1}$ multi-element external standard. 7 elements were detected from the 55 elements analysed (supplementary information Table SI.3.10). Participants CZ8, CZ9 and CZ10 samples have not been included due to incomplete sample sets as they were unable to provide a full set of samples as a consequence of not be able to extract blood from the collection site.

3.3.5.1 Multivariate Analysis

The 7 detected elements (variables) against participant plasma samples (observations) made up the plasma data set. The s-plot generated following PLS-DA indicated that three elements could be potential elemental biomarkers of stress, out of the seven elements that were detected (Figure 3.11). PCA of the three elements, Mg, P and K, did not produce a distinct separation between the three observation groups. However, it may be argued that the baseline observations have a higher PC[2] value than the recovery 1 observations which in turn have a higher PC[2] value than the recovery 2 observations (Figure 3.11). This model produced two significant principal components exhibiting 99.1% [PC1] and 0.70% [PC2] of total explained variance of the data set for physical stress.

Although there is no evidence of clear separation in human plasma obtained from the seven participants following physical stress, there is an indication of a slight trend when examining the baseline, recovery 1 and recovery 2 observations. The majority of baseline observations appear at a high PC[2] value and this value tends to decrease in observation following the stress intervention. This suggests a change in the elemental content of human plasma obtained following physical stress, although it is very slow. Observations from recovery 2 are in a separate band with the lowest PC[2] values. When viewing the baseline and recovery 2 values in isolation, it is clear there are two definite bands suggesting changes in elemental concentrations following physical stress. However, due to the slow response to the intervention participant elemental concentrations did not recover to baseline levels as a consequence of limited experiment time.

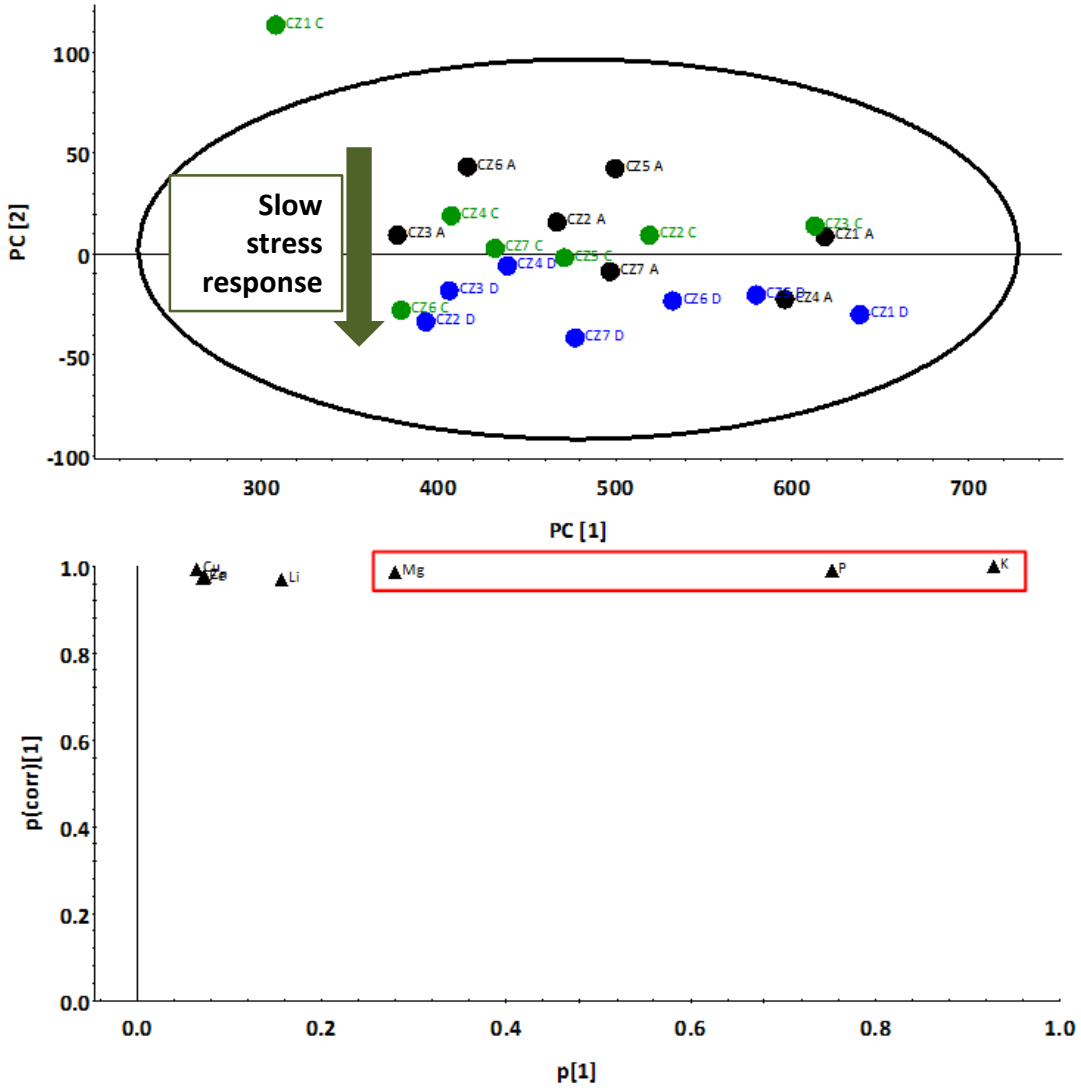


Figure 3.11. Top: PCA scatter plot of three stress sensitive plasma components identified from PLS-DA s-plot on 7 observations each from the baseline (black dots), recovery phase 1 (green dots) and recovery phase 2 (blue dots) measurements from seven male participants. Bottom: S-plot generated from the modelled covariance (P(corr)[1]) and modelled correlation (p[1]) of the PLS-DA from the concentration of 7 plasma sensitive elements (variables) from 24 saliva samples (observations). The three variables highlighted (red square) were found to provide the highest magnitude (concentration) of variables in the data set and greatest reliability (P(corr)[1]axis) of the variables in the data set, indicating potential elements sensitive to exercise

3.3.5.2 Descriptive Statistics

From the 7 detected elements in participant plasma, large concentrations of Mg, P and K were observed whilst relatively high concentrations of Li were present. Lower concentrations, but still relatively high, of Fe, Zn and Cu were also detected. The largest changes occurred for K, Cu and Zn. Between phases A and C changes of -11, -10 and -11% were calculated for K, Cu and Zn, respectively. Between the two recovery phases C and D, K increased by 20% whilst the second biggest change between these two phases was for Cu with an increase of 6%. For all elements, overall change between phase A and D was less than 10%. Due to little change between these three phases, it may be that elemental

concentrations in plasma both change and recover so quickly that it cannot be detected within the time frame set for collecting samples. Alternatively, changes in elemental concentrations are so slow that more time may have been needed between sample collections.

Table 3.6. Concentration (ng.mL-1), change (%) and paired t-test (P) results of 7 detected elements found from participant plasma collected from three phases. Bold values indicate statistical significance at 95%

Phase		Li	Mg	P	K	Fe	Cu	Zn
A	μ	4004	12910	94917	134503	793	683	891
	σ	1364	1377	15719	27714	284	146	275
C	μ	3711	12506	86462	119445	805	614	793
	σ	1551	1398	17675	33797	115	150	266
D	μ	3725	12327	86358	143169	835	649	814
	σ	1728	1216	18218	26162	262	144	237
Between phases								
Δ (%)	A to C	-7	-3	-9	-11	1	-10	-11
	C to D	0	-1	-0	20	4	6	3
	A to D	-7	-5	-9	6	5	-5	-9
t test (P)	A to C	0.696	0.663	0.433	0.478	0.686	0.470	0.462
	C to D	0.938	0.858	0.983	0.295	0.990	0.747	0.817
	A to D	0.672	0.213	0.155	0.471	0.559	0.581	0.488

The results of ANOVA suggested no statistical evidence of significant differences at 95% as the calculated F value (1.15) was lower than the critical value of F (3.07) for all elements and phases (Appendix Table 8.7). The paired t-test data, using normalised data (log_{10}), confirmed that no differences in elemental concentrations were observed at 95% (Table 3.6). H_0 for this test was that no difference in elemental concentrations observed prior to and following physical stress, was accepted for all tests conducted. Hence there was no statistically significant evidence of differences in elemental concentrations between phases from human plasma.

3.3.6 Identifying major elemental contributors from multivariate analysis using global elemental data from physical stress

A data set with values collected from skin, saliva, urine and plasma samples, all analysed by ICP-MS, was constructed. This data set was used to construct MVA models in order to observe the major elemental and matrix contributors in physical stress. Prior to modelling all data sets were prepared to exclude all incomplete datasets.

Thirty three variables, from the four analysed biological samples, against fifteen observations were used to construct the element data set with a total of fourteen elements used. PLS-DA modelling was initially performed using normalised elemental concentrations

(variables) from phases A, C and D of the study after which an S-plot was constructed (Figure 3.12).

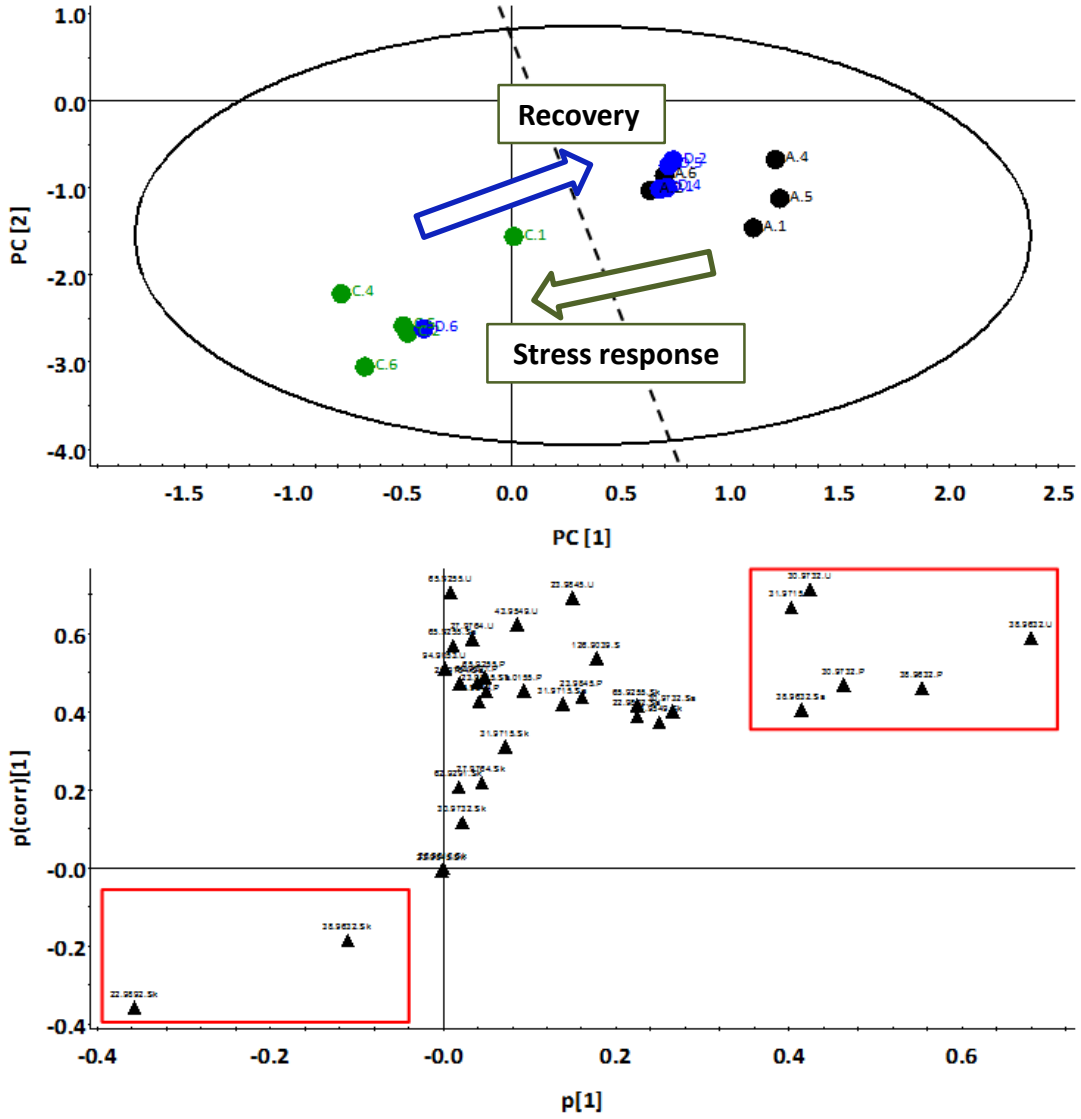


Figure 3.12. Top: PCA scatter plot of eight stress sensitive components identified from PLS-DA s-plot on 5 observations each from baseline (black dots), recovery phase 1 (green dots) and recovery phase 2 (blue dots) measurements from five male participants. 100% sensitivity was obtained immediately following the stress phase and 100% specificity was obtained for baseline measurements. Bottom: S-plot generated from the modelled covariance (P(corr)[1]) and modelled correlation (p[1]) of the PLS-DA from the concentrations of 33 elements and biological samples (variables) from 15 participants and phases (observations). Eight highlighted variables (red square) were found to provide the highest magnitude of variables in the data set and greatest reliability (P(corr)[1]axis) of the variables in the data set, indicating potential sensitivity to exercise.

Eight variables, chosen from the PLS-DA s-plot, were used to create an unsupervised model. The eight variables were 22.9892.Sk, 30.9732.P, 30.9732.U, 31.9715.U, 38.9632.P, 38.9632.Sa, 38.9632.Sk and 38.9632.U. PCA of these eight elements displayed a separation between baseline (phase A) and recovery 1 (phase C) observations with two principal components displaying 59.5% [PC1] and 22.1% [PC2] of the total explained variance of the data set for physical stress yielding 100% sensitivity for recovery 1 measurements and 100%

specificity for the baseline measurements. Interestingly, phase D measurements (blue dots) are clustered near the baseline measurements, indicating that for these chosen variables the participants have recovered by the time the study is completed. The major elements chosen, from the supervised model, in this model were from the elements P (30.9732) and K (38.9632) indicating these may be major elements in predicting physical stress.

3.3.7 Conclusion for the elemental analysis of skin, saliva, urine and plasma obtained from physical stress

A total of 14 elements were detected from skin, saliva, urine and plasma as a result of physical stress. 4 of these detected elements (Mg, P, K and Zn) were detected in all four biological samples while 3 elements (Na, Si and S) were found in three of the biological samples (skin, saliva and urine). Additionally, Ca was detected in skin and urine while Cu was found in skin and plasma. Al was only observed in skin, I in saliva, Mo in urine while Li and Fe were detected in plasma. Whilst independent findings of elements are observed the fact that many of these elements are found in multiple biological samples indicates that biological pathways, between the examined samples, are connected. Zn is known for its healing properties in the immune system therefore it is no surprised to see this element present in all four biological samples following physical stress requiring the need for immediate repair in the body. K is essential in the body for electrolyte balance, blood pressure and heart function, assists the nervous system, releases energy from protein and helps delivery oxygen to the brain promoting efficient cognitive function. Mg is known to maintain normal nerves and muscle function, supports the immune system, keep the heart beat steady, assist in maintaining strong bones, regulates blood glucose and aids the production of energy and protein. P, located in the bones and teeth, is essential for the growth and repair of body cells and tissues. It is found in the body as phosphate (important for ATP – energy), DNA and helps maintain the pH of the blood. Na, found in three of the biological samples, controls blood pressure and regulates the function of muscles and nerves while Fe, found in plasma only, distributes oxygen around the body via cells. Due to the connection of biological pathways in the body it is clear why these elements are recovered in most if not all four of the biological samples studied for this experiment. Therefore, physical stress changes in elements found in one of the biological samples at a specified location in the body can also be an indication of the changes occurring in a different part of the body.

Although clear separation is achieved using MVA, it is difficult to identify the actual compounds of which these elements are being produced as the data, obtained from all biological samples analysed in this chapter by ICP-MS, only details elemental concentrations.

To gain a better understanding of what compounds these elements may be derived from, samples should be analysed using other mass spectrometry techniques which provide greater information on larger molecules, e.g. using TD-GC-MS to investigate VOCs or UPLC-IM-MS to examine small molecules, respectively.

3.3.8 Identifying major biomarkers of physical stress from multivariate analysis using global elemental, VOCs and small molecules data

The chemical changes in human saliva, as a result of physical stress, can exhibit a number of things as both the mouth and the body are integral to each other. Signals of diseases such as mouth lesions (HIV), ulcers (Crohn's disease), pale/bleeding gums (blood disorder), changes in the tooth (anorexia/bulimia) and presence of compounds (drugs detected in saliva) are observed within the mouth. Furthermore, oral system conditions can be an effect of the immune system. Biological interactions between oral conditions and medical conditions are not fully understood. However, research indicates it is certainly linked.

Saliva was collected from three of the experimental phases and analysed by ICP-MS (elements), TD-GC-MS (VOCs) and UPLC-IM-MS (small molecules). VOC analysis and data processing was carried out by Helen Martin (personal communication) and small molecule analysis and data processing was carried out by Aditya Malkar (personal communication) (as referred to in Appendix VII Publications). The data set was constructed using data obtained using these techniques provided by the aforementioned people. This data set was used to create MVA models which in turn were used to observe major contributors for physical stress.

The saliva data set were generated to include only common features from all three techniques used to analyse the matrix. Saliva was only collected from three phases; therefore no phase B samples were included. Samples from participants CZ3 (absent for element and VOC), CZ7 (absent from element) and CZ10 (absent from element) were also excluded. Data for participants CZ1, CZ2, CZ4, CZ5, CZ6, CZ8 and CZ9 were included. Each sample matrix for each participant was normalised to 1 using the exact method of normalisation used previously. Variables were renamed to enable easy identification of element, VOC or small molecule, and then sorted in order of name.

Thirty variables; eight from elements; fourteen from VOCs; and eight from small molecules, against twenty one observations were used to construct the saliva data set. PLS-DA modelling was initially performed on the normalised data set of variables from the three selected phases of the study resulting in an S-plot modelled from PLS-DA (Figure 3.13). Ten

variables, chosen from the PLS-DA s-plot, were used to produce an unsupervised model exhibiting separation between baseline (phase A) and recovery 1 (phase C) observations with two principal components displaying 53.8% [PC1] and 13.2% [PC2] of the total explained variance of the data set for physical stress.

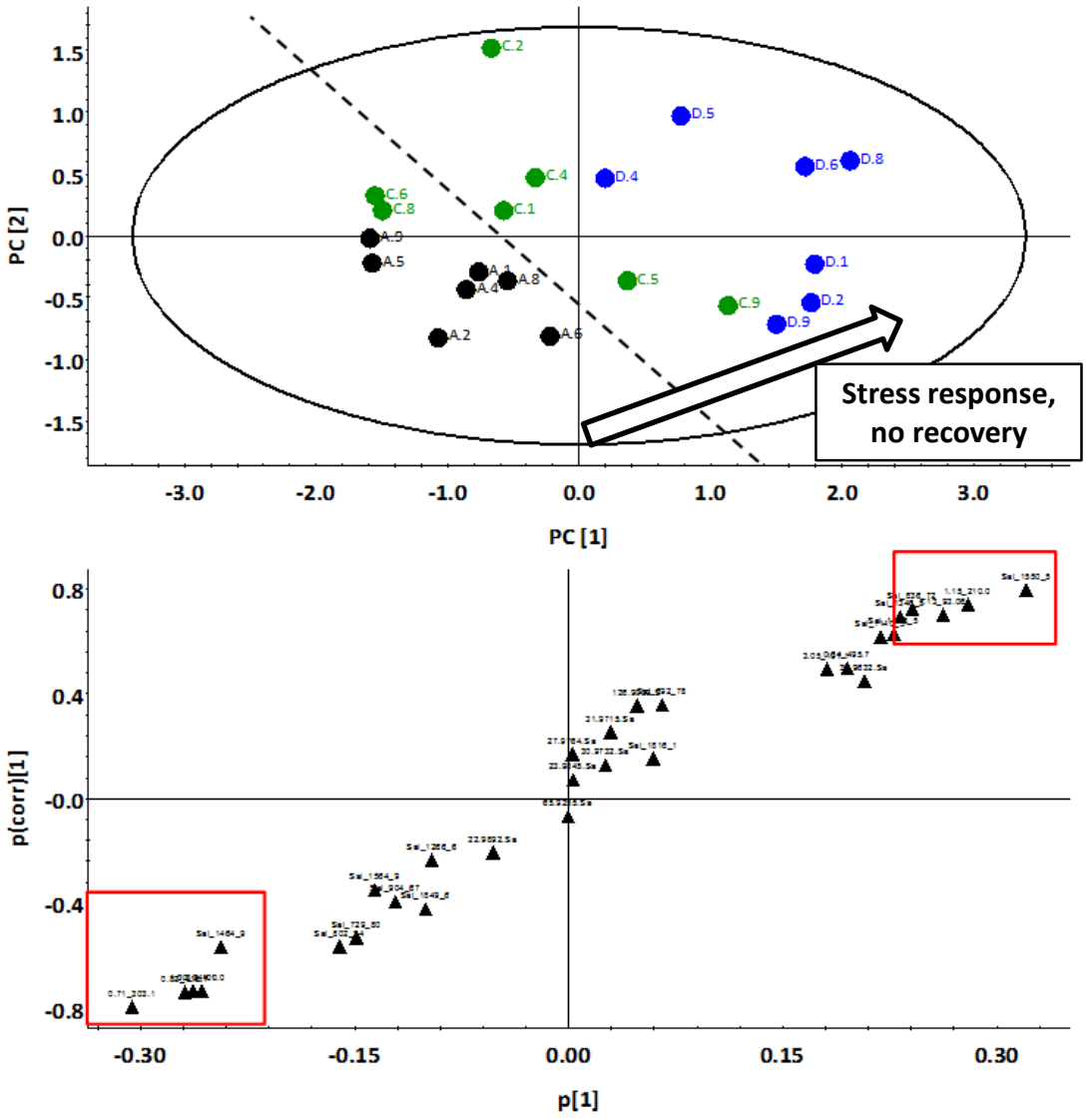


Figure 3.13. Top: PCA scatter plot of eight stress sensitive components identified from PLS-DA s-plot on 7 observations each from baseline (black dots), recovery phase 1 (green dots) and recovery phase 2 (blue dots) measurements from seven male participants. 71.4% sensitivity was obtained immediately following the stress phase and 100% specificity was obtained for baseline measurements. Bottom: S-plot generated from the modelled covariance (P(corr)[1]) and modelled correlation (p[1]) of the PLS-DA from 30 variables (8 elemental, 14 VOC and 8 small molecule data) from 21 participants and phases (observations). Ten highlighted variables (red square) were found to provide the highest magnitude of variables in the data set and greatest reliability (P(corr)[1]axis) of the variables in the data set, indicating potential saliva sensitivity to exercise.

The ten variables chosen were: 5 up regulated from s-plot Sal_1464_94_107_121_135, 0.71_303.1447, 0.82_418.7209, 1.00_244.1285 and 1.31_100.0750, and 5 down regulated Sal_836_72_57_43, Sal_1346_57_82_55_67, Sal_1550_57_82_55_67, 1.13_93.0689 and 1.15_210.0502. 71.4% sensitivity was observed for recovery 1 measurements and 100%

specificity was observed for baseline measurements. Intriguingly, phase C observations in Figure 3.13 move further away from phase A observations and in turn, phase D observations move further from phase C observations indicating that the recovery from physical stress in element, VOC and small molecules found in saliva, is very slow.

Identification of these potential biomarkers proved difficult due lack of financial resource and time. Although accurate mass has been obtained the instrument libraries contained a number of possible structures for each marker. Purchasing every standard suggested and testing the eluted peak of the standard against the observed peaks did not prove very practicable considering the limited instrument time. Also, this method of validation could see well over one hundred standards purchased for very little initial promise. Ideally, this study would be repeated with at least forty more participants with the results obtained compared against those observed from this study. In the event where the same masses of stress markers were discovered, standards should be purchased and the mass identified to give a known compound. However, if the libraries were unable to determine possibilities for these markers, it may be entirely possible that a new chemical compound of stress was discovered.

3.4 Conclusion

This experiment studying the chemical effects of physical stress exhibits that the method to detect biomarkers of stress can be successful. Samples from human skin, saliva, urine and plasma samples were obtained prior to and following physical stress. 10 elements (Na, Mg, Al, Si, P, S, K, Ca, Cu, Zn) were detected in human skin. The highest concentrations were found for Na (7.5 $\mu g.mL^{-1}$) and K (11.5 $\mu g.mL^{-1}$) following physical stress with significant changes in elemental concentrations observed for eight of these elements (not observed for Si and Zn). For skin samples obtained during the final recovery phase (over 30 minutes after the end of the physical stress exercise) element concentrations were found to recover back to baseline levels observed in the pre-intervention samples. The high element concentrations immediately following the stress intervention was expected due to higher sweat levels observed during the stress period. 8 elements (Na, Mg, Si, P, S, K, Zn, I) were detected in human drool saliva. Highest concentrations of elements were observed in pre stress saliva for Na (21.6 $\mu g.mL^{-1}$), P (26 $\mu g.mL^{-1}$) and K (63.5 $\mu g.mL^{-1}$) with significant changes presented for S and I. Evidence of a slow decrease in element concentrations in saliva was found following physical stress. Only I concentration decreased immediately following stress. S and I concentrations were found to decrease in the second recovery phase sample when compared to the pre stress samples. No statistically significant differences were observed for any element between the two recovery phases. 8 elements were also detected in human urine (Na, Mg, Si, P, S, K, Ca, Zn, Mo). The highest concentrations were found in the second recovery phase samples for the elements P (1050 $\mu g.mL^{-1}$), S (2150 $\mu g.mL^{1}$) and K (5500 $\mu g.mL^{-1}$). A general decrease in all elemental concentration, except Si and Zn, was found following stress after which concentrations returned to the levels observed prior to physical stress. Significant differences were exhibited for all elements except Mo. 7 elements were detected in human plasma (Li, Mg, P, K, Fe, Cu, Zn) with the highest concentrations found in samples collected prior to the physical stress exercise for Mg (13 $\mu g.mL^{-1}$), P (90 $\mu g.mL^{-1}$) and K (130 $\mu g.mL^{-1}$). However, no statistically significant differences were found between any of the three phases for which the samples were collected. Consequently, future experiment should investigate elemental changes in whole blood following the physical stress intervention rather than examining blood plasma. There is a possibility more information will be available in whole blood compared to plasma due to the removal of many components of whole blood to yield plasma.

Many elements were detected due to their important functions within the body with significant differences in concentrations observed following the stress intervention. For example, K has a role to assist the nervous system hence following physical stress the elements were observed in all four biological samples investigated. Similarly, Zn plays a role maintaining a healthy immune system as does Mg. P is found in bones and teeth yet it is essential for growth and repair of body cells and tissues which are affected following physical stress while Na regulates the functions of muscles and nerves. This evidence strengthens claims that biological pathways as in fact linked and where one pathway affected by physical stress can also cause others to be influenced too.

Data from 8 elements, 14 VOCs and 8 small molecules were also used to create a saliva data set to enable identification of possible biomarkers of physical stress. MVA of the saliva data set showed that a wealth of information can be derived from analysing simultaneously collected saliva, from participants following physical stress, using three difference techniques. Although the definite identification of these biomarkers was not achieved due to financial cost and time restraints, it is possible to purchase and analyse standards to confirm possible formula's identified from instrument libraries.

Future studies could use a similar method to collect and analyse samples using at least a further forty participants, with the modifications of a longer experiment time in order to allow participant stress levels return to those observed at baseline levels as well as collecting and analysing whole blood instead of plasma for elemental changes. In the event similar biomarkers are recovered using all three techniques, known standards should be purchased and analysed for identification of the biomarkers of stress. These could then be used to produce drugs that counter act these biomarkers and seek to investigate clinical trials on humans who are thought to be stressed.

4 A study into the effect of emotion stress on the elemental content of saliva as measured by ICP-MS

Emotional stress is another form of stress with little to no physical measure, as per the studies in the previous chapters. The objective of this study was to identify elemental biomarkers from elemental profiles obtained by ICP-MS in human saliva, following emotional stress and is part of a wider study by Ran *et al.* (Appendix VII, Reference 3). Saliva samples were collected prior to and following an intervention designed to replicate a trapped human in a building for twenty four hours causing emotional stress. H_0: There was no difference in the elemental composition of human saliva obtained prior to and following stress i.e. H_0: $ESR_{PRE} = ESP_{POST}$.

This study was devised by Ran Huo in collaboration with Paul Thomas. All elemental saliva samples were collected and analysed by the author at the Centre of Analytical Sciences at Loughborough University. The motive to collect samples from this study was to identify elemental changes in human saliva collected from an emotional stress intervention and compare any differences in the elemental changes in saliva collected from previous studies, namely from physical stress.

4.1 Experimental Outline

One volunteer aged 50 years old participated in this study. On the morning of sample collection the participant was asked not to wash with any fragranced soap. Saliva sample collection lasted a maximum of 10 minutes before and after the 24 hour intervention. A safe and reproducible simulator of a person trapped inside a collapsed building was designed, built and tested by members of this research group. A range of environmental conditions to simulate collapses in different climates could be generated. Three sub-systems were also used; i) an environmental chamber, providing a buffer reservoir of purified air at a specified humidity to the void chamber, ii) a void simulator, for a large participant (120 kg and 2m) to lie in, iii) a collapsed building simulator, air passed through here from the void chamber. The collapsed building simulator presented generic elements of a collapsed building including reinforced concrete and glass. Whole saliva was collection before and after one participant was *trapped* in a simulated building collapse for 24 hours. Elemental analysis of the saliva was performed by ICP-MS.

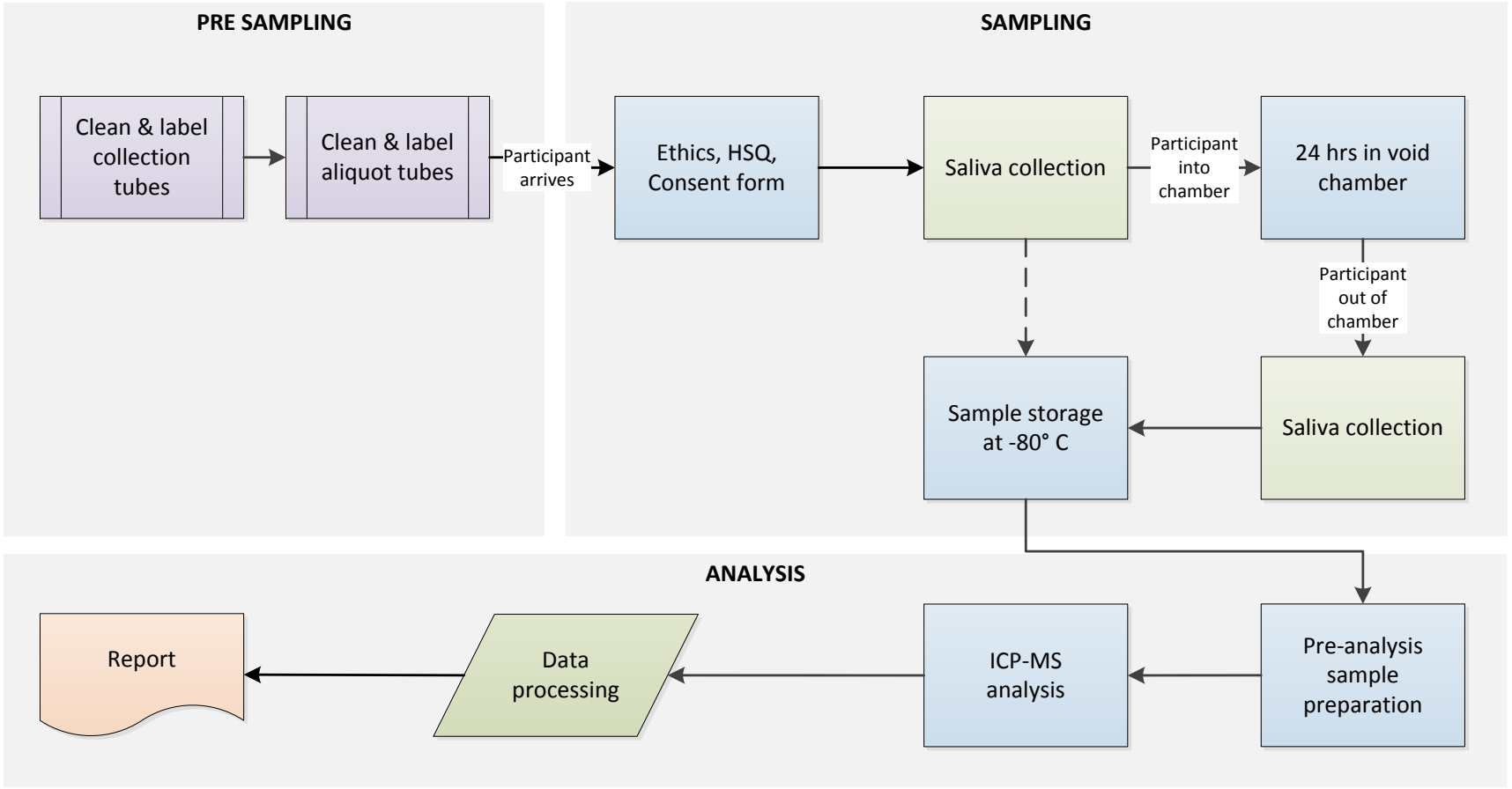


Figure 4.1. Experimental procedure for saliva sampling

4.1.1 Collection Tubes Preparation

Two 30 ml glass tubes (Fisher Scientific, Loughborough, UK) were pre cleaned by rinsing with DI H_2O followed by a 5 minute soak with 5 ml of 2% HNO_3. These were placed on a shaker (ELMI Skyline shaker, Riga, Latvia) for 5 minute at a tilt of 5°. The HNO_3 was appropriately disposed and 5 ml of DI H_2O was added. The vials were left for 5 minutes on a shaker at a tilt of 5° after which the DI H_2O was disposed and the tubes were left to air dry for 2 hours. One tube was labelled 'Pre' and the other 'Post'.

4.1.2 Storage Tubes Preparation

Six 2ml cryotube vials (VWR International Ltd, Leighton Buzzard, UK) were also labelled. Three were to be used to aliquot the saliva samples provided prior to the participant entering the chamber and three to be used once 24 hours had elapsed and the participant came out of the chamber.

4.1.3 Saliva Collection

Prior to entering the void chamber the participant was asked to provide 5 ml of mouth drool (saliva) into the 'Pre' labelled tube by tilting their head forward and letting saliva pool at the front of their mouth and then drooling this into the 20 ml tube. Three 1 ml aliquots of the drool were then placed into pre cleaned 2 ml cryotubes. These replicates were labelled 'Pre A', 'Pre B' and 'Pre C' and stored in a 4°C fridge.

After 24 hours had elapsed, the subject was assisted out of the void chamber and was asked to provide 5 ml of mouth drool (saliva) in the 'Post' labelled tube. As with the pre saliva samples, three 1 ml aliquots of drool was then placed into the pre cleaned 2 ml cryotube vials labelled 'Post A', 'Post B' and 'Post C'.

4.1.4 Sample Analysis

Samples were stored at -80°C until the day of analysis, for a maximum of 28 days. Samples were left to thaw out at room temperature for a minimum of 60 minutes. The six aliquots were then thoroughly mixed, for homogeneity, with the aid of a vortex mixer. For each sample, 1 ml of saliva was mixed with 4 ml 2% HNO_3 in a 15 ml polypropylene centrifuge tube (Elkay, Hampshire, UK) (Figure 4.1). Twenty six elements were analysed using ICP-MS (described in Chapter 1 section 1.3.5) with a randomised analysis order for all samples (Figure 4.1). Three internal replicates were analysed for all samples (six saliva samples plus a solvent blank). Significance testing was performed to discover whether any statistical evidence of elemental differences between samples existed.

4.2 Results and Discussion

Elemental analysis of six saliva samples (three pre intervention replicates and three post intervention replicates) and a solvent blank was performed by ICP-MS. No MVA was performed on the data set in this chapter as all samples were provided by one individual.

4.2.1 Elemental Profiles

The elemental profile (Figure 4.3) shows that the majority of elements with the highest saliva concentration can be found with elements lower in mass than 50 Daltons. Although Sn (117.9011 Da) and I (126.9039 Da) do show high concentrations in saliva, the blank samples also exhibit the highest concentrations for these samples.

Table 4.1. Average concentration ($ng.mL^{-1}$) and relative standard deviation for the nine statistically significant (95%) elements including relative change, F- and T-test (probability) score

Element	Pre	Post	Change (%)	F_{calc}	T_{calc} (P)
Na	120276 (26.4)	52 (27.6)	-99.96	0.000	0.003
I	107567 (23.3)	10363 (5.40)	-90.37	0.001	0.003
Mn	44 (36.6)	12 (14.6)	-72.40	0.024	0.027
Fe	105 (34.2)	37 (14.1)	-64.54	0.042	0.032
P	214235 (14.0)	151849 (10.9)	-29.12	0.467	0.034
Si	340 (9.40)	541 (13.0)	37.24	0.346	0.011
Mg	3807 (20.3)	7318 (12.2)	47.98	0.853	0.007
As	2.7 (50.0)	7 (18.5)	63.19	0.995	0.014
Sr	10 (27.6)	46 (10.4)	77.52	0.527	0.000

4.2.2 Descriptive Statistics

ANOVA was performed to determine if any significant differences (95%) were present between i) three replicate pre intervention samples ii) three replicate post intervention samples and iii) the pre and post intervention groups. The results for these tests can be observed in the appendix Table 8.10. Two-factor without replication ANOVA was performed on the pre and post intervention replicate samples. Replicate pre and post intervention samples showed no evidence of significant difference with calculated F values of 1.624 and 1.340, respectively. This was lower than the critical F value of 3.183 suggesting no evidence of significant differences (95%) between the replicate samples.

ANOVA (two factor with replication) was performed to find if there was any statistical evidence of significant difference in elemental concentration between the two groups. The calculated F value of 2.328 obtained was bigger than the F critical value (degrees of freedom 25) of 1.612 therefore evidence was present to suggest that the elemental concentrations between the pre and post intervention groups were statistically significant.

It was not clear from the data which of these elements were statistically significant from just the ANOVA testing. As a result, t-tests were carried out to determine whether element concentrations between the pre and post intervention groups were statistically significant (95%). Nine elements showed statistical significance between the pre and post samples from the twenty six elements that were analysed. These were Na, I, Mn, Fe, P, Si, Mg, As and Sr. The calculated probability of the t-test (2 tailed, equal variances) can be found in Table 4.1. for these nine elements. Statistical information for all 26 elements can be found in the Appendix Table 8.11. T_{calc} (P) of less than 0.05 shows evidence of statistical significance between the pre and post intervention samples.

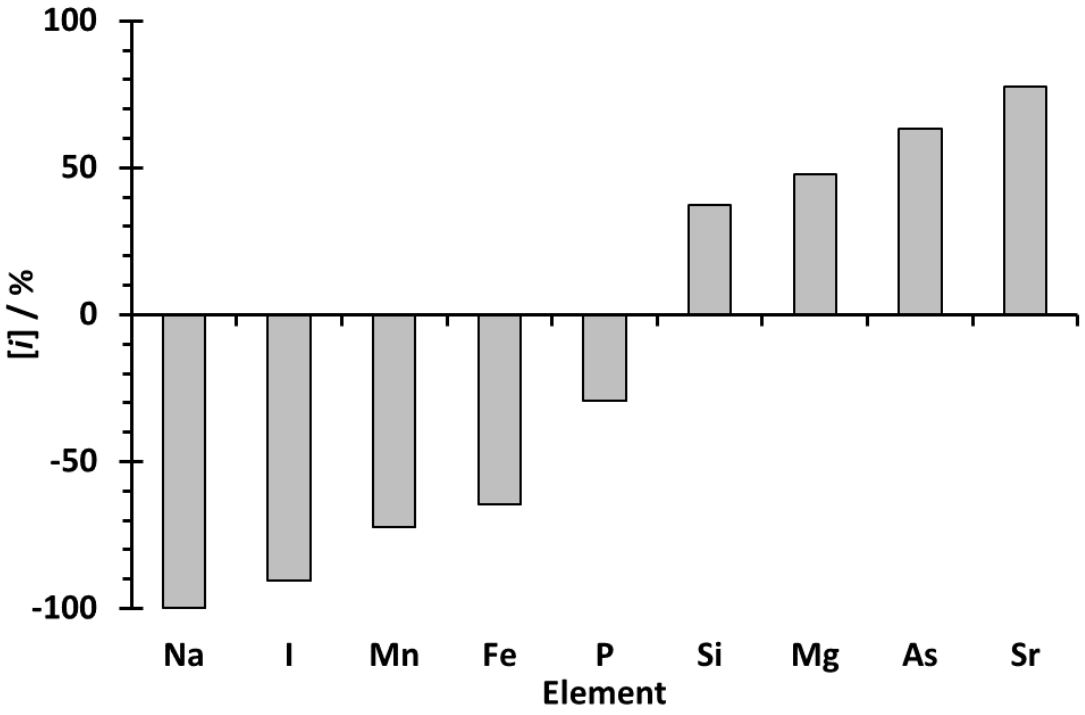


Figure 4.2. Relative changes between the pre and post intervention samples for the nine statistically significant elements

The element concentrations ($ng.mL^{-1}$) for pre and post intervention samples for these nine elements can be found in (Table 4.1). The highest elemental concentrations in saliva, from the nine elements where statistical significance was evident, were P, Na and I. In the pre-intervention samples the highest elemental concentration is found for P, approximately 210 $\mu g.mL^{-1}$, which almost doubles that of the next highest concentration which is Na, approximately 120 $\mu g.mL^{-1}$. A high concentration of I is also present in the pre-intervention sample with a concentration approximately 100 $\mu g.mL^{-1}$. Also in this sample, low concentrations were found for As, Sr, Mn, Fe and Si, at 2.7, 10, 44, 105 and 340 $ng.mL^{-1}$, respectively. Mg was also present at a concentration of nearly 4000 $ng.mL^{-1}$(Figure 4.2).

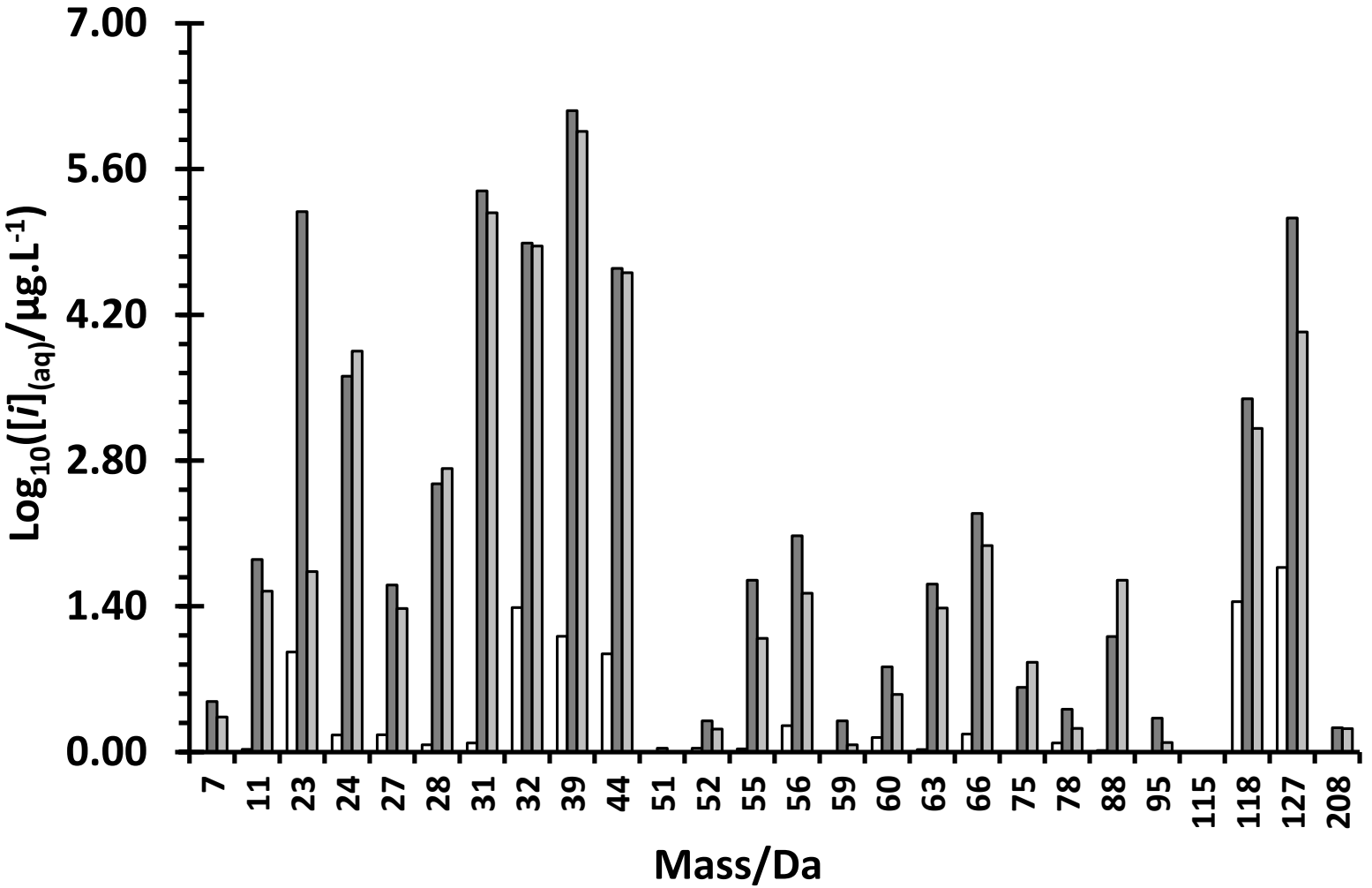


Figure 4.3. Saliva profile showing intensity of elemental masses from 0-210 Da for Blank B sample (white), Pre intervention B sample (dark grey)and Post intervention B sample (light grey)

Five elements decreased in concentration, whilst four elements increased in concentration, between the pre and post intervention samples (Figure 4.2). Na, I and Mn displayed the largest decreases with a relative change of 99.9, 90.4 and 72.4 %, respectively. The relative decrease for Fe was 64.5% whilst that for P was 29.1%. The largest relative increases were observed for Sr (77.5%) and As (63.2%) whilst Mg (47.98%) and Si (37.2%) also displayed a relative increase between the pre and post intervention samples.

The increase in concentration of Sr is an interesting and unexpected one. However, previous studies into the effect of toothpaste show that Sr is used in toothpaste for very practical reasons. Surdacka *et al*. studied the effect of Sr toothpaste on artificially decalcified human enamel and found that regular tooth brushing with Sr supplemented toothpaste increased the Sr content in exposed enamel, after three months compared to the levels observed at the baseline, which can be advantageous in the prevention of cariogenesis.[90] West *et al*. studied pain reduction in a variety of toothpastes and found that Sr based toothpastes resulted in the reduction of pain in the short term.[91]

4.3 Conclusion

Whole saliva was collected from 1 human participant trapped in a replication of a building collapse for 24 hours in order to investigate the effects of emotional stress. Saliva was collected using a drool method immediately before and after the participant entered the void chamber for 24 hours, during which the participant did not eat and experienced sleep deprivation. Two saliva samples were collected and elemental analysis was performed by ICP-MS.

Sixteen elements were detected in both sets of samples. Statistically significant changes (95%) were observed for nine elements in whole saliva for twenty-four hours of a human in an entrapped space with no food or water due to emotional stress and sleep deprivation. H_0 was rejected (i.e. H_0: $ESR_{PRE} \neq ESP_{POST}$) as elemental concentrations in saliva obtained from a human participant who had been trapped in a chamber for twenty four hours with no food or drink, were affected.

The largest changes were found for Na (-99.9%) and I (-90.4%) which both decreased by over 90% baseline concentration. Concentration decreases were also found for Mn (-77.4%), Fe (64.5%) and P (-29.1%) while Sr (77.5%), As (63.2%), Mg (48.0%) and Si (37.2%) increased in concentration. However, it is unclear whether these changes are due to emotional stress or deprivation of sleep and food due to the saliva obtained from only one participant. It is known that there is a concentration change in teeth Sr to prevent certain diseases and these contributed to the concentration change in Sr in saliva due to leeching of Sr from the teeth to saliva. Further experiments with more participants should be conducted before any comparisons between emotional stress and physical stress can be achieved.

5 The Liquid Skin Pen

The aim of this work was to design and engineer a non-invasive skin sampling device in collaboration with Unilever Plc. The long term goal was to discover biomarkers of stress from human skin using the device. The skin sampling device was produced to target the sampling of several skin sites of the human body in order to better understand the chemistry of these sites. Samples obtained were analysed by ICP-MS, ESI-MS or nESI-MS to deduce elemental and small molecule content. Results obtained from the device could be used to create or improve existing skin products produced by Unilever Plc.

At the time of writing no procedures exist for the real-time, site-specific sampling and analysis of small molecule and elements from human skin. The Liquid Skin Pen (LSP) provides the first step for a device that can be developed to collect site specific real time samples from the skin. This would be advantageous compared to current methods such as the use of adhesive pads, which require precious time between sample collection and data evaluation; because online analysis of samples would ensure that skin conditions (such as dermatitis) may be diagnosed instantly.

The experiments in sections 5.4 and 5.5 were devised and carried out by the author. Section 5.6 outlines an experiment that was devised and carried by the author and Dr Corrinne Burns. The experiment stated in section 5.7 was devised by Svetlana Riazanskaia and carried out in collaboration with the author and Dr Corrinne Burns. The final experiment in section 5.8 was devised by the author and carried out with the assistance of Dr James Reynolds. Sample analysis was performed by the author with assistance from Dr Corrinne Burns and Dr James Reynolds.

5.1 Design and construction of the Liquid Skin Pen

The LSP was engineered by Peter Carroll at (Unilever PLC, Bebington), in consultation of the design by the author, Dr. Corrinne Burns (Loughborough University), Prof. C.L.Paul Thomas (Loughborough University) and Svetlana Riazanskaia (Unilever PLC, Bebington). The outer casing of the device is constructed of white acetal, the inner tubing is made from PEEK (grade GF 30, ID = 0.254 mm OD = 1.5875 mm) and the liquid inlet sterile hypodermic needles (Becton, Dickinson, Plymouth, UK) are constructed of stainless steel. Material used to construct the device can be found in Table 5.1 and a schematic diagram of the LSP design can be seen in Figure 8.1.

Table 5.1. LSP material

Part	Material
LSP body	Acrylic
Sampling base	PEEK GF30
Sample delivery holder	Acetal
Sample delivery vial	Clear glass, silanized
Sample delivery needle	Hypodermic
Sample delivery tube holder	PEEK GF30
Sample collection holder	Acetal
Sample collection vial	Clear glass, silanized
Sample collection needle	Stainless steel
Sample collection tube holder	PEEK GF30
Transport tubes	PEEK GF30
Top block	White acetal
Peristaltic pump outlet	PEEK GF30

5.2 Target Sample Sites

Whilst the primary focus on the early stage of development for the LSP was use on the human skin, initial studies (sections 5.4 and 5.5) were performed on a non-biological surface using manganese (Mn). Mn was used as this was available in the laboratory as well as only Mn having a single isotope (^{55}Mn). It must be worth noting that ^{55}Mn is not an ideal element to experiment with due to possible polyatomic interferences of KO, NaS, ArN, ClO, ArOH, ArN and ArO. Therefore data analysis had to be conducted thoroughly ensuring these interferences did not affect the amount of Mn observed in the results.

Table 5.2. Sites samples and delivery solvent used with the LSP

Section	Sample Type	Sample Site	Sampling solvent
5.4	Mn sample	Surface of nitrile glove	0.1M EDTA
5.5	Mn sample	Surface of nitrile glove	0.1M EDTA, 2% HNO_3 DI H_20
5.6	Human Skin	Volar & plantar	0.1M EDTA
5.7	Human Skin	Axilla, dorsal, plantar & volar	0.1M EDTA
5.8	Human Skin	Dorsal & Plantar	0.1M EDTA

The surface of a power free nitrile glove (Barber Health Care Limited, Leyburn, UK) was chosen in order to replicate human skin prior to sampling on human volunteers. During these initial tests (sections 5.4 and 5.5), any issues that arose with sampling using the LSP could be resolved in order to improve the efficiency of sampling when later using human volunteers.

Figure 5.1. Mn sample dried for 30 minutes on a nitrile glove

Four skin sites were targeted and sampled using the LSP when using human volunteers. Sample sites included the axilla (AP), dorsal (TF), plantar (BF) and volar (AE) (Figure 5.2). These sites were targeted as the device was initially used for diagnostic purposes with the hope that it could eventually be developed to finding stress biomarkers of human skin leading to the production of new skin products, for Unilever Plc., helping to aid other diseases such as dermatitis.

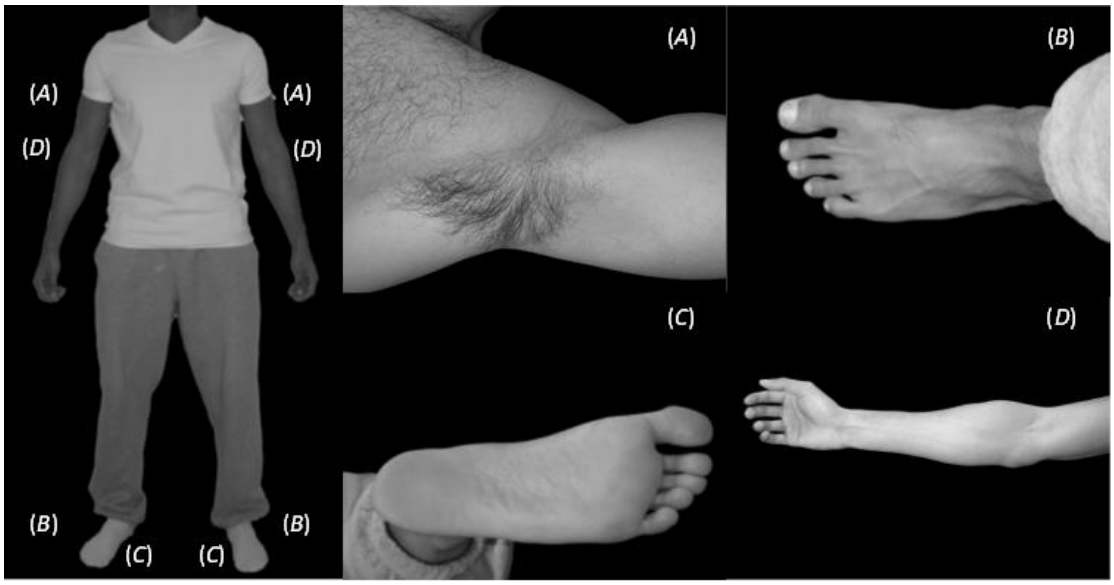


Figure 5.2. The four sites sampled using the LSP (A) Axilla (B) Dorsal (C) Plantar (D) Volar

5.3 Sample Collection using the Liquid Skin Pen

Samples were collected from a target area using negative pressure which was achieved by means of a peristaltic pump. For the experiments in sections 5.4, 5.5 and 5.6, the peristaltic

pump (Cole Parmers' Masterflex C/L) was set at 200 rpm. For the sections 5.7 and 5.7.2 the peristaltic pump (Watson-Marlow 120U with Bioprene tubing of 1.6 mm wall, 1.6 mm bore) was set at 32 rpm.

A screw capped *delivery* vial (National Scientific, Loughborough, UK), containing approximately 1.5 ml of sampling solvent, was placed in the green LSP *delivery* vial holder and an empty screw capped *collection* vial (National Scientific, Loughborough, UK) in the red LSP *collection* vial holder (Figure 5.3). The peristaltic pump was switched on and the pump tubing was connected to the LSP peristaltic pump outlet. The base of the LSP was then gently applied to the surface of the sampling area forming a closed system in order for sampling to begin via negative pressure.

Immediately after each site was sampled the vial of the collected sample was recapped with a new, clean screw cap with an un-pierced rubber septum in order to avoid air contamination.

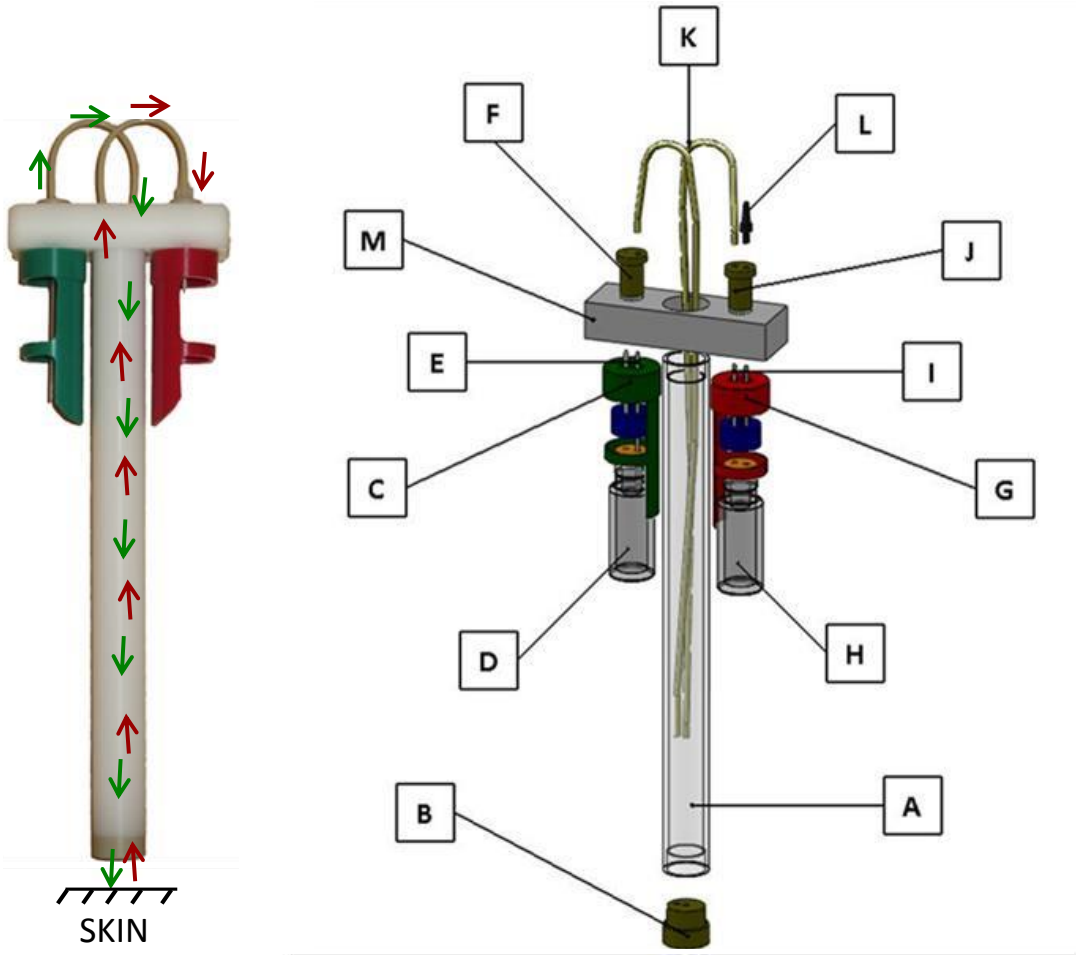


Figure 5.3. LSP parts; A. LSP body; B. Sampling base; C. Delivery vial holder; D. Delivery vial; E. Delivery needle; F. Delivery tube connector; G. Collection vial holder; H. Collection vial; I. Collection needle; J. Collection tube connector; K. Sample transport tubes; L. Peristaltic pump connection; M. Top block

Samples were obtained in less than 60 seconds once the face of the sampling base covered the target sampling area. Sampling solvent would flow from the delivery vial through the body of the LSP via the PEEK tubing to the target area, where sampling of the surface area took place, after which the sample solution was transported through the second PEEK tube and would drop out into the collection vial. Once the transfer of the sample solution had come to an end, the peristaltic pump was switched off and the collection vial was removed from the LSP vial holder. The screw caps (containing two pierced holes through the septum from the hypodermic needles) of the sampled delivery and collection vials were then replaced with new screw caps (VWR, Leighton Buzzard, UK) without any pierced septa. This was carried out in order to protect the sample from any potential impurities in the air during storage. Furthermore, both the delivery and collection vials (clear silanized glass I-D™, National Scientific) were weighed without caps prior to and following sample collection to obtained the transfer efficiency of the sampling solvent.

During sample collection, the LSP would work best when the target sample surface was as flat as possible and facing away from the floor with the LSP in an upright position with the sampling base facing (parallel) the floor.

5.3.1 Vial Preparation

Pre sample collection vial measurements

Vials were prepared on the morning of sample collection. 1.5 ml sampling solvent was added to the delivery vials, weighed* and then capped. The remaining empty vials (collection vials) were also weighed* and then capped.

**Vials were always weighed without caps*

Post sample collection vial measurements

Delivery and collection vials were weighed and masses recorded immediately after sample collection. Vial caps were removed during weighing and new, clean caps were screwed onto the vials.

5.3.2 LSP Pre Sample Collection Pen Cleaning

For sections 5.4, 5.5 and 5.6 approximately 4ml of sampling solvent was flushed through the LSP using a hypodermic needle and syringe via the sample holes located at the base of the LSP (Figure 5.3 part B). 2 ml was pushed through each of the sample holes and the sampling solvent travelled through the PEEK sample transfer tubes exiting through needles located at either the sample delivery or collection side of the LSP. Following the sampling solvent flush,

further LSP flushes, using new syringes and needles, were performed using 4 ml of DI H_2O and finally the sampling solvent again.

For sections 5.7 and 5.8 all LSP's were cleaned together using the sample method above. However, the solvents used to clean the LSP's were (in order) 4 ml 0.5% v/v $NH_{3(aq)}$, 4 ml DI H_2O, 4 ml 0.5% v/v $CH_3COOH_{(aq)}$, 4 ml DI H_2O and 4 ml 50% v/v $EtOH_{(aq)}$. Once all the LSP's had been cleaned, they were placed in a clean foil tray and were dried in a vacuum oven (Binder, Tuttingen, Germany) at 40°C overnight. Once drying was completed, capped vials were placed in both the delivery and collection vial holders to protect the sampling needles from air contamination.

The final solvent used to clean the LSP was EDTA, prior to collecting the LSP pen blank for the next sample site.

5.3.3 LSP Pen Blank Sample Collection

LSP Pen blanks were collected for each study for each of the LSP's used, prior to sample collection. For studies where only one LSP was available, pen blanks were collected prior to sample collection from each participant.

Capped sample vials were placed in both the delivery vial holder and the collection vial holder of the LSP. Approximately 1 ml of sampling solvent was flushed through each hole in the sampling base of the LSP using sterile syringes (Becton Dickinson, Plymouth, UK) and sterile hypodermic needles (Becton Dickinson, Plymouth, UK). Sampling solvent which had passed through the LSP and was present in both the delivery and collection vials were then mixed together, using a syringe, into the pre weighed vial.

5.3.4 Inter Participant Cleaning of the LSP

For studies involving human volunteers, the LSP was cleaned before and after collecting samples from each participant. To ensure that all the sampling solvent had been removed from the LSP, several injections of DI H_2O were forced through the LSP from the base of the device using a the procedure outlined for LSP pen cleaning in section 5.3.2. The LSP was then dried overnight in a vacuum oven (Binder) at 40°C, for 5 hours. Once the LSP was cleaned, pre-cleaned empty vials with screw caps were placed in the delivery and collection holders of the LSP. The sampling and delivery needles of the LSP at both ends would pierce through the rubber septum of the vial caps ensuring they would not be exposed to the outside air for a significant period of time.

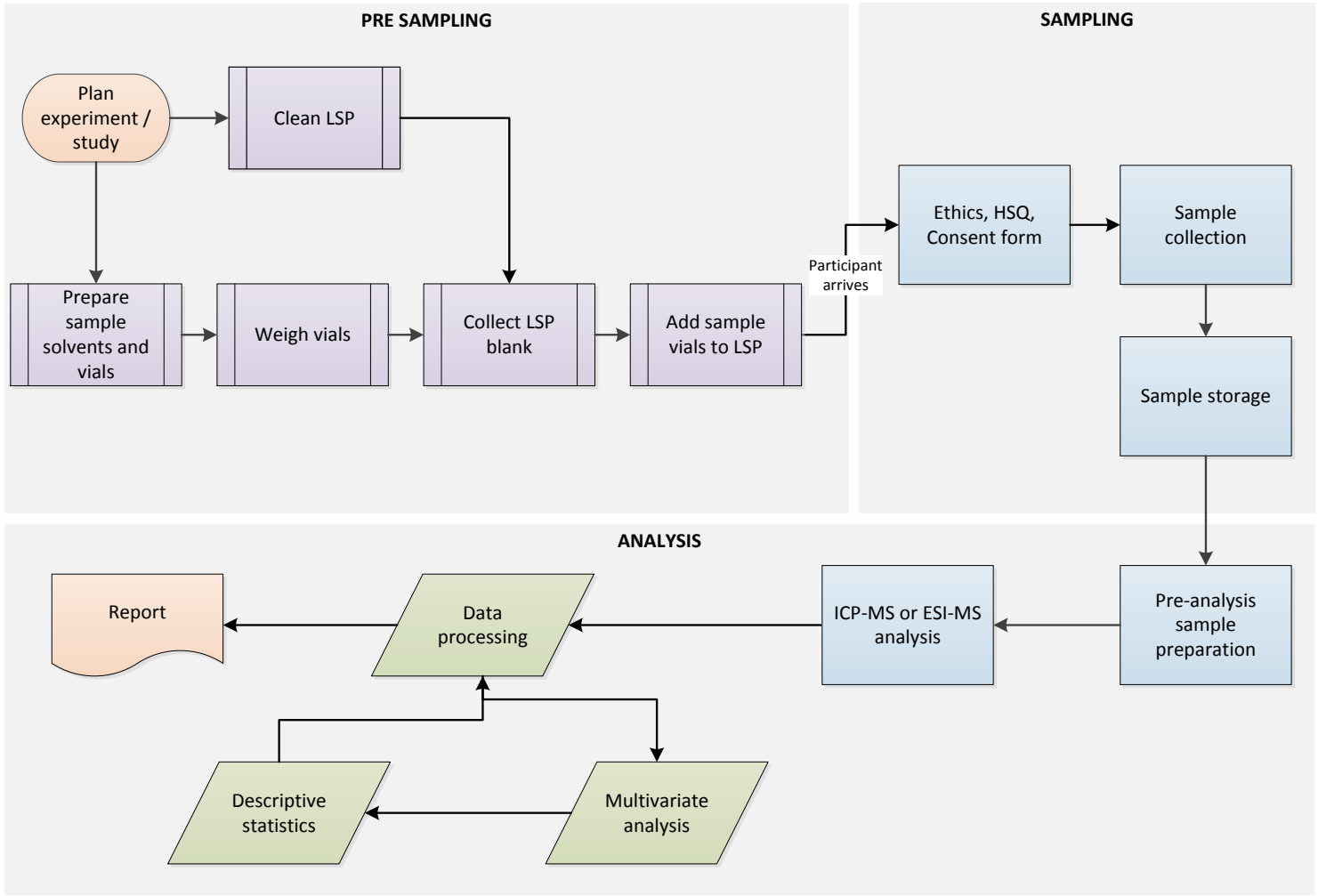


Figure 5.4. Workflow for experiments sampling with the LSP

5.4 An experiment to determine the efficiency of elemental extraction using the LSP

As detailed in Chapter 1, there is little information regarding the elemental content of human skin as a result of different types of stress placed on the body. The objective of this experiment was to investigate if the LSP was able to extract one drop of 10 µg.ml^{-1} Mn from a nitrile surface using a sample solution of 0.1M EDTA. Furthermore, should extraction of Mn be successful what amount of recovery could be achieved in the primary sample and subsequent wash samples. H_0: there was no difference in the amount of Mn recovered from a nitrile glove using the LSP, between surfaces that has been spiked with Mn (Sample ID: B) compared to one that has not (Sample ID: A). i.e. *H_0: [A] = [B]*.

5.4.1 Experimental Outline

One drop of 10 µg.mL^{-1} Mn was weighed and added onto the surface of a fresh (out of the box), unused nitrile glove (Barber Health Care Limited, Leyburn, UK) using a 20 µg pipette (Gilson Inc., Middleton, USA). The drop was allowed to sit on the surface and dry for 30 minutes prior to sample collection. The first sample (sample B) was collected from the target area followed by three subsequent wash samples (B1, B2 and B3). All samples were collected from the exact same spiked target area of the glove. Samples were stored in a 20°C freezer immediately following collection.

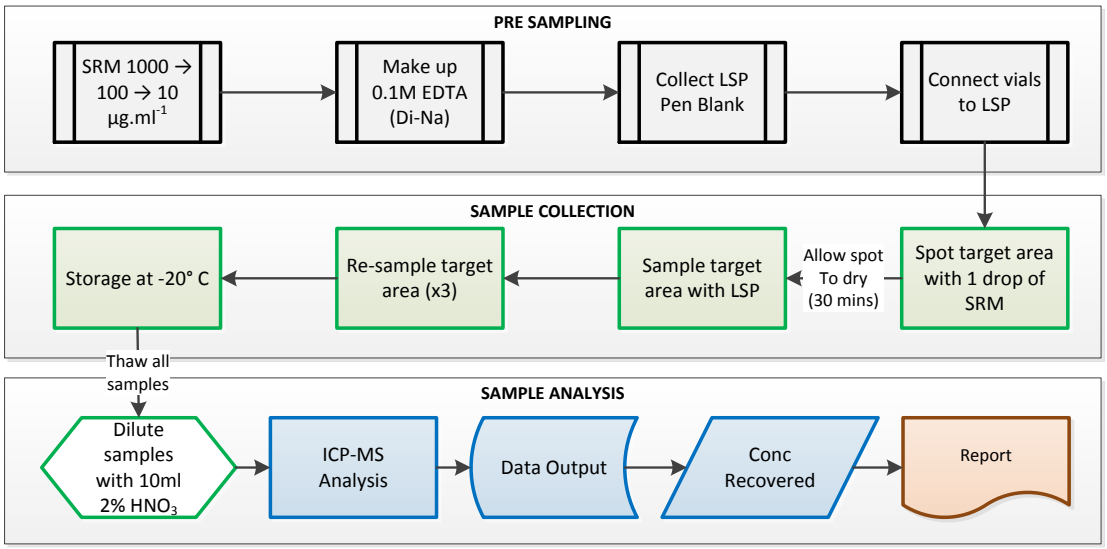


Figure 5.5. Experimental procedure for LSP recovery testing

5.4.1.1 Sample Analysis

All samples were analysed on the subsequent day of collection and were allowed to thaw at room temperature for 30 minutes after which they were homogenised using a vortex mixer (Scientific Industries Inc., NY, USA). For all samples, 1 ml of collected sample was diluted

with 10 ml 2% HNO_3 in 15 ml fresh, unused, polypropylene centrifuge tubes (Elkay Ltd, Basingstoke, UK).

Elemental detection of Mn was performed using an ICP-MS (as described in Chapter 1 section 1.3.5). A 10 $\mu g.mL^{-1}$ Mn sample was also analysed in order to semi-quantify sample concentrations. Three replicates were taken for each sample analysed. Elemental concentrations in the sample were semi quantified by using the ratio of the intensity obtained for the known Mn concentration against intensity obtained for all samples. Additionally, relative Mn recovery was determined from each of the collected samples.

5.4.2 Results and Discussion

A total of 8 samples were successfully analysed on the same day. Samples obtained from the LSP included a LSP solvent blank, one Mn sample and three Mn wash samples. Also analysed were two solvent blanks, which consisted of DI H_2O and 2% HNO_3, and a 10 $\mu g.mL^{-1}$ Mn sample.

Table 5.3. Concentration of samples (µ), blank subtracted samples (µ (BS)), relative standard deviation (RSD) and recovery of Mn in samples obtained using the LSP. n = 3

Sample	Concentration ($ng.mL^{-1}$)		RSD	% Recovery
	µ	µ (BS)		
A	2.71		9.4	
B	7.24	4.53	6.1	41.8
B1	4.18	1.47	4.3	13.5
B2	3.44	0.73	5.6	6.7
B3	3.06	0.35	6.2	3.2
C	0.34		9.4	
D	0.32		10.3	

The LSP successfully extracted the Mn sample from the target surface (Table 5.3). 2.71 $ng.mL^{-1}$ of Mn was extracted using the LSP from the unspiked target surface and used as the baseline value. However, once a drop of the Mn sample was spiked on the target surface a concentration of 7.24 $ng.mL^{-1}$ Mn was obtained. Subsequent washes exhibited a decrease in the amount of Mn extracted from the surface with 4.18, 3.44 and 3.06 $ng.mL^{-1}$ found in washes 1, 2 and 3, respectively. The amount of Mn attained in the solvent blanks was less than the amount obtained from the blank. Figure 5.6 illustrates the reduction in the concentration of Mn following each wash. In the first sample, following the surface spike with the Mn (B), the highest concentration of Mn is acquired when compared to subsequent samples where the Mn concentration gradually decreases from wash 1 (B1) to wash 2 (B2) and finally wash 3 (B3). This data suggests that although the LSP is able to extract a

significant amount of Mn in the first sample on the target surface, it does not achieve 100% recovery, as proven by the Mn concentrations found in the wash solutions. By removing the amount of Mn found in the blank sample from the spiked sample as well as subsequent wash samples, the Mn concentration achieved are 4.53, 1.47, 0.73 and 0.35 ng.mL^{-1} for the initial spiked sample, wash sample 1, 2 and 3, respectively.

5.4.2.1 Recovery of Mn using the LSP

The theoretical concentration of Mn expected in the final sample analysed by ICP-MS can be calculated using a predicted 100% recovery. There were three transfer phases for Mn, each step diluting the sample analyte more than the previous phase. The three phases are; step 1, where a known amount of Mn sample was added to the target surface; step 2, a known amount of sampling solvent was used to sample the target surface; and step 3, dilution of the collected LSP sample with a matrix suitable for ICP-MS analysis.

Theoretical Concentration

Step 1

An average mass of one drop of Mn used was 0.0179 g (σ = 0.0001, n = 3) with a measured concentration 10.0087 µg.mL^{-1}. Therefore, an estimated 17.9 mg Mn was added onto the target surface of an unused, fresh latex glove.

Step 2

Using the LSP, 1.5 ml EDTA was sampled and collected over the target area containing Mn resulting in the first dilution.

$$C_1 \, X \, V_1 = C_2 \, X \, V_2 \qquad \text{(Equation 5.1)}$$

Where C_1 = Initial concentration
V_1 = Initial volume
C_2 = Final concentration
V_2 = Final volume

Using equation 5.1, if C_1 = 10.0087 µg.mL^{-1}, V_1 = 0.0179 and V_2 = 1.5 then C_2 = 119.2 µg. L^{-1}.

Step 3

1 ml of sample collected was diluted with 10 ml 2% HNO_3, in a 15 ml polypropylene tube, prior to ICP-MS introduction. Re-arranging equation 5.1, the theoretical 100% recovery concentration (C_2) can be calculated. C_2 = 0.01083 µg.mL^{-1}, when C_1 = 0.1192 µg.mL^{-1}, V_1 = 1ml and V_2 = 11 ml.

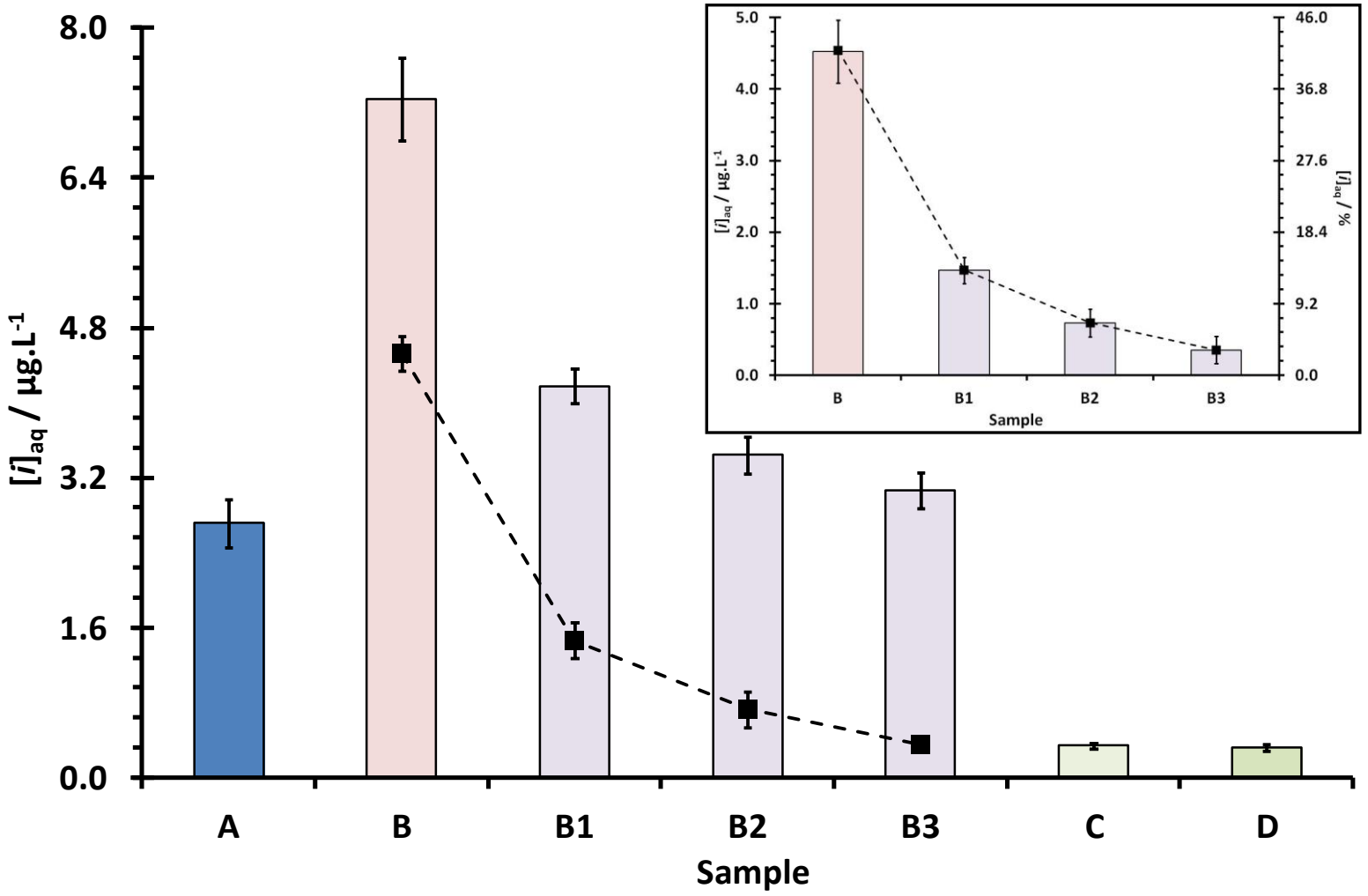


Figure 5.6. Mean concentration ($[i]_{aq}$/ng.mL^{-1}) of Mn (σ = 5%) from samples collected using the LSP. Bars = Concentrations (ng.mL^{-1}) Line = Blank subtracted concentrations (ng.mL^{-1}). Insert: Bars = Blank subtracted concentration (ng.mL^{-1}), Line = Recovery

$$\% \, Recovery = \frac{Experimental \; concentration}{Theoretical \; concentration} \; X \; 100 \qquad \text{(Equation 5.2)}$$

Therefore, the theoretical concentration of 100% recovery would be 10.83 µg.mL^{-1}. Table 5.3 shows the calculated relative recovery of Mn for each sample, obtained using Equation 5.2. The initial sample contained 41.8% (4.53 ng.mL^{-1}) of the original Mn sample whereas recoveries of 13.5 (1.47 ng.mL^{-1}), 6.7 (0.73 ng.mL^{-1}) and 3.2% (0.35 ng.mL^{-1}) were obtained for the subsequent washes, 1, 2 and 3, respectively. This is illustrated in Figure 5.6.

H_0 was rejected as a difference in the amount of Mn recovered from a nitrile glove, using the LSP, between a spiked surface and blank surface (H_0: *B ≠ Blank*) was discovered. Although it was proved that the LSP can be used to detect a sample of Mn on the surface of a nitrile glove, multiple washes are needed to extract as much Mn as possible. However, it is worth noting that the first sample taken with the LSP did achieve the highest recovery.

Issues encountered included sample uptake stalling from the delivery vial. This was solved by replacing the cap and septum on both the delivery vials and re-placing them into the vial holders. It was important to ensure that vials were inserted into the vial holders vertically in order to avoid bending the hypodermic needles at both the delivery and collection vial sites of the LSP.

5.5 An experiment to determine the most efficient solvent for metal extraction using the LSP

EDTA has extensive medical, industrial, agricultural and engineering applications and is a widely used chemical compound found in cosmetic preparations and cleaning, processed foods, skin care and personal care products. High purity nitric acid is commonly used for metal analysis in mass spectrometry whereas water is used as part of everyday life. Due to its function as a hexadentate ligand and chelating agent, EDTA has the ability to capture metal ions such as Fe^{3+} and Ca^{2+}. After being bound to EDTA, metal ions remain in solution but exhibit reduced reactivity. At a very low pH (very acidic conditions), the fully protonated H_2EDTA^{2+} form is prevalent whereas at very high pH (very basic conditions) the fully deprotonated form $EDTA^{4-}$ is predominant. $EDTA^{4-}$ usually binds to metal cations through its four carboxylates and two amines with the resulting coordination adopting an octahedral geometry. Metals are extensively enveloped by EDTA resulting in the suppression of their catalytic properties. Since complexes of $EDTA^{4-}$ are anionic they are likely to be highly soluble in water.

The aim of this experiment was to identify which of three readily available solvents were best suited as the LSP sampling solvent (S_S) in order to recover a known concentration of Mn from a target surface. In addition, a further objective was to investigate the efficiency of the LSP. The three solvents investigated were 0.1M EDTA, 2% HNO_3 and DI H_2O. H_0: there was no difference in the amount of Mn recovered from a nitrile glove, using the LSP, using the three sampling solvents. i.e. H_0: S_S *(x)* = S_S *(y)* = S_S *(z)*.

5.5.1 Experimental Outline

1 LSP was used to collect a total of 15 samples. This included 4 samples (1 sample from the spiked area of Mn and the 3 subsequent wash samples from the exact location) and 1 LSP pen blank for each of the 3 sampling solvents used. One drop of 100 µg.mL^{-1} Mn was initially weighed after which it was added onto the surface of a fresh (out of the box), unused nitrile glove using a 20 µL pipette. The drop was allowed to sit on the surface and dry for 30 minutes.

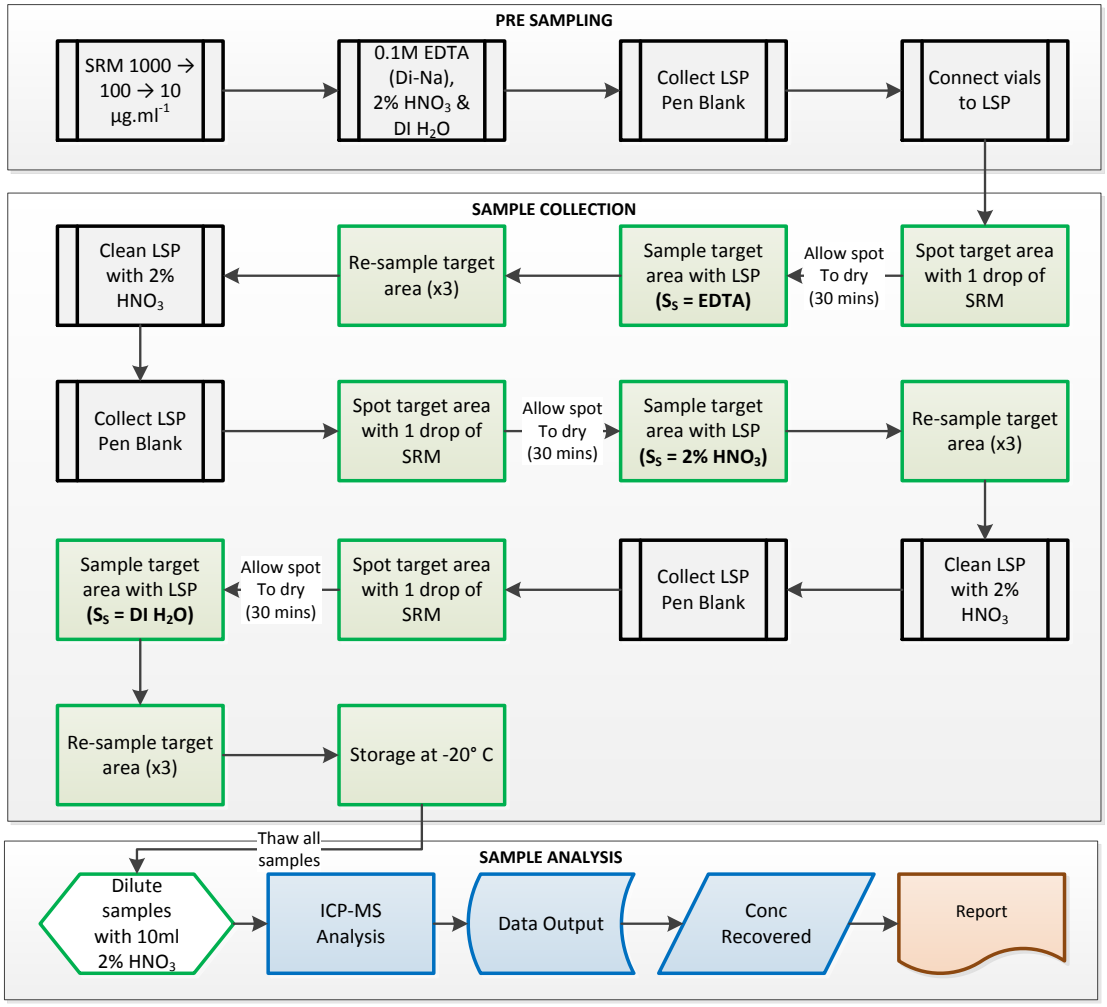


Figure 5.7. Experimental procedure for sample solution testing using the LSP for solvent comparison

A clean, fresh out of the box nitrile glove was used for each solvent sample test (3 nitrile gloves used in total). EDTA samples were collected first, followed by 2% HNO_3 samples and finally DI H_2O samples. Each sample was collected within 60 seconds once the face of the sampling base covered the target sample area. All samples were collected on the same day and stored at -20°C until the day analysis.

5.5.1.1 Sample Analysis

Samples were removed from storage after 2 days, allowed to thaw at room temperature for 30 minutes and then homogenised using a vortex mixer. For all 15 samples, 1 ml of collected sample was diluted with 4 ml 2% HNO_3 (1:5 dilution) in 15 ml fresh, unused, polypropylene centrifuge tubes (Table 5.3).

All samples were analysed on the same day they were removed from storage. Elemental detection of Mn was performed using ICP-MS (Chapter 1 section 1.3.5). Each sample was analysed in triplicate and sample concentrations were semi quantified by taking the ratio of intensity obtained for the known Mn concentration of 100 $\mu g.mL^{-1}$ against those obtained for samples. Comparing the concentrations of samples with the known concentration of the drop enabled the recovery to be calculated as well as the sampling efficiency of the LSP (sampling solvent transfer from the delivery to collection vial) (supplementary information Table SI.5.2).

5.5.2 Results and Discussion

A total of 15 samples obtained using the LSP, plus a sample containing 100 $\mu g.mL^{-1}$ Mn which was analysed for semi quantitation purposes, were successfully analysed on the same day. Mn was successfully extracted from the target surface using all three delivery solvents of interest.

5.5.2.1 Recovery of Mn using the LSP

Theoretical 100% recoveries for each sample were calculated using the same procedure as that used in section 5.4.2.1. Once again, three phases were conducted. Step 1, where a known amount of a Mn sample was added to the target surface. Step 2, a known amount of sampling solvent was used to sample the surface using the LSP. Finally step 3, in order for the sample to be analysed using ICP-MS analysis, the collected sample was diluted with a suitable matrix.

Theoretical Concentration

STEP 1

The average mass of one drop of Mn used was 0.010 g (σ = 0.0001, n = 5) with a calculated concentration of 90.7877 µg.mL^{-1}. Therefore, an estimate of 0.010 g of Mn was added onto the target surface of an unused, fresh latex glove.

STEP 2

Approximately 1.5m l of sampling solvent was delivered and collected over the target area, where the drop of Mn had been placed, using the LSP. Using Equation 5.1, the concentration of this dilution was calculated for each sample (Table 5.4). C_1 = 90.7877, V_1 = 0.010, V_2 = 1.5, and C_2 = unknown. For most samples this concentration was approximately 60 ng.mL^{-1}.

STEP 3

Finally, 1 ml of sample collected was diluted with 4 ml 2% HNO_3, in a 15 ml polypropylene tube, enabling ICP-MS introduction. In this step, C_1 = 60 ng.mL^{-1}, V_1 = 1 ml, V_2 = 4 ml and C_2 was the unknown concentration for a 100% theoretical recovery. These calculated values can be viewed in the step 2 column of Table 5.4. For most samples, 100% recovery would yield a concentration of approximately 11 ng.mL^{-1} Mn.

The experimental concentrations for each of the samples are also shown in Table 5.4. For the first sample using the LSP for each sampling solvent, Mn concentrations of 6.5, 4.6 and 2.9 ng.mL^{-1} were observed for the 2% HNO_3, EDTA and DI H_2O, respectively. Subsequent samples using the LSP, for each sampling solvent, showed a decrease in concentration obtained showing that the first sample obtained extracts the largest amount of Mn. For the first sample, the highest concentration achieved was with the LSP using 2% HNO_3 as the sampling solvent, followed by EDTA and finally DI H_2O. This can be seen in Figure 5.8 along with the decreasing trend in concentration for subsequent samples using each of the three sampling solvents. Very little of the Mn sample was extracted from the target surface following the first two samples using the LSP.

5.5.2.2 Recovery

Using Equation 5.2 and both the theoretical and experiment concentrations calculated previously, the relative recovery for each sample was calculated (Table 5.4). For the primary sample, the highest recovery of Mn was observed when using the sampling solvent 2% HNO_3

with a recovery of 55.7%. The next highest achieved was when using EDTA as the sampling solvent and then DI H_2O with relative recoveries of 39.9% and 18.6%, respectively. For the first wash sample, the highest recovery was attained when using 2% HNO_3 (32.6%) as the sampling solvent followed by EDTA (19.7%) and finally DI H_2O (12.0%). The LSP pen blank recovery when using 2% HNO_3 as the sampling solvent was 12.4%, for EDTA was 4.3% and DI H_2O was 1.2%. The last two wash samples using the LSP were lower in recovery compared to each of the blanks for the relevant sampling solvents and this trend is shown in the insert in Figure 5.8.

Table 5.4. Calculated theoretical ($C_{THEORETICAL}$) and experimental concentrations ($C_{EXPERIMENTAL}$) ($ng.mL^{-1}$ Mn) and recovery (%) of Mn obtained from the three sample solutions collected using the LSP

Sample	$C_{THEORETICAL}$		$C_{EXPERIMENTAL}$		Recovery (%)
	Step 1	Step 2 (final)	μ (n=3)	RSD (%)	
EDTA					
Blank	57.36	11.86	0.514	4.75	4.3
Sample (B)	56.31	11.46	4.573	3.28	39.9
Sample wash 1 (B1)	58.48	11.81	2.328	4.41	19.7
Sample wash 2 (B2)	57.84	11.88	0.516	1.13	4.3
Sample wash 3 (B3)	55.27	11.26	0.482	3.25	4.3
			Total (B to B3)		**68.2**
2% HNO_3					
Blank	60.18	12.02	1.486	4.45	12.4
Sample (B)	58.49	11.60	6.465	3.40	55.7
Sample wash 1 (B1)	60.69	12.43	4.057	3.00	32.6
Sample wash 2 (B2)	61.21	12.52	1.480	3.90	11.8
Sample wash 3 (B3)	58.95	12.18	1.474	4.02	12.1
			Total (B to B3)		**112.3**
DI H_2O					
Blank	57.29	11.55	0.144	3.33	1.2
Sample (B)	77.71	15.48	2.871	3.98	18.6
Sample wash 1 (B1)	60.63	12.25	1.473	5.72	12.0
Sample wash 2 (B2)	51.81	10.49	0.100	3.67	1.0
Sample wash 3 (B3)	59.44	11.97	0.088	3.10	0.7
			Total (B to B3)		**32.3**

Intriguingly, the total Mn recovery from the samples and washes yielded 112.3% when using 2% HNO_3 as the sampling solvent whereas total recovery when using the sampling solvents EDTA and DI H_2O were calculated to be 68.2 and 32.3%, respectively. If it is considered that only a maximum of 100% should be theoretically recovered, there is an extra 12.3% recovery of Mn using 2% HNO_3 as the sampling solvent. A number of factors should be considered to explain this extra recovery. The 2% HNO_3 samples were the second batch of samples collected, using the same LSP, following sample collection using EDTA as the sampling solvent. It may have been possible that the LSP may not have been thoroughly cleaned and there were contaminants of the Mn from the previously collected batch resulting in memory

effects of Mn. The high blank sample recovery could point to this. Another factor could be extraction of traces of indigenous Mn from the nitrile glove itself.

5.5.2.3 Significance Testing

ANOVA was performed to investigate significant differences between the first samples obtained from the LSP of each of the three solvent samples used (Appendix Table 8.13). The calculated probability of F (346.02) was much larger than the critical value of F (5.14), suggesting evidence of significant differences between the first sample collected using each sampling solvent.

Table 5.5. Intensity (counts per second), mean (μ), standard deviation (σ) and relative standard deviation (%) of Mn signal from the three sample solutions collected using the LSP. Probability score for test of variance (F) and difference sample means of the previous sample collected (T) are also given where values P < 0.05 show evidence of statistically significant differences in the mean

Sample	Intensity (cps)					RSD (%)	F (P)	T (P)
	1	2	3	μ	σ (n = 3)			
EDTA								
Blank	51959	54551	57144	54551	2593	4.75		
Sample (B)	500856	469013	484935	484935	15921	3.28	0.05	**3.35E-04**
Sample wash 1 (B1)	257746	246854	235963	246854	10891	4.41	0.64	**7.24E-05**
Sample wash 2 (B2)	54716	55332	54100	54716	616	1.13	0.01	**6.88E-06**
Sample wash 3 (B3)	52739	49421	51080	51080	1659	3.25	0.24	**4.92E-02**
2% HNO_3								
Blank	164553	150520	157537	157537	7017	4.45		
Sample (B)	708881	685541	662201	685541	23340	3.40	0.17	**2.49E-04**
Sample wash 1 (B1)	443086	430187	417287	430187	12900	3.00	0.47	**3.82E-04**
Sample wash 2 (B2)	150832	163078	156955	156955	6123	3.90	0.37	**8.75E-05**
Sample wash 3 (B3)	150060	162615	156337	156337	6278	4.02	0.98	9.09E-01
DI H_2O								
Blank	14732	15748	15240	15240	508	3.33		
Sample (B)	292296	316554	304425	304425	12129	3.98	0.00	**2.06E-06**
Sample wash 1 (B1)	147289	156225	165162	156225	8936	5.72	0.70	**1.24E-04**
Sample wash 2 (B2)	10567	10179	10955	10567	388	3.67	0.00	**9.40E-06**
Sample wash 3 (B3)	9322	9033	9611	9322	289	3.10	0.71	**1.33E-02**
					Between sample 1 of delivery matrix			
					EDTA & 2% HNO_3		0.64	**2.51E-04**
					EDTA & DI H_2O		0.73	**9.81E-05**
					2% HNO_3 DI H_2O		0.43	**1.50E-05**

A t-test to compare the two experimental means was conducted to investigate whether these differences occur between the first samples obtained from each of the sampling solvent. Table 5.5 shows the probability of the t-test and implies there is evidence of significant differences between the first samples obtained using the LSP for all three sampling solvents. The probability obtained between the first sample when using the solvent samples EDTA and 2% HNO_3 was 0.000251, much less than the 0.05 threshold, and thus there is evidence present of statistically significant differences. Evidence of statistically

significant differences (95%) were also present between sampling solvents EDTA and DI H_2O (T = 0.0000981) and 2% HNO_3 and DI H_2O (T = 0.000015). As shown in Table 5.5, t-tests between results from consecutive samples showed statistically significant differences in all cases except between the final 2 washes of the samples using 2% HNO_3.

5.5.2.4 Efficiency

Using the data collected for these samples, the relative efficiency of the LSP was determined by taking the amount of solvent sampled over the target surface (solvent collected) and subtracting from this the initial amount of solvent delivered (delivery solvent), from the delivery vial, over the target sample area. Multiplying this by 100 produces the relative efficiency (equation 6.3). The relative efficiency for all samples obtained can be found in supplementary Table SI.5.2.

$$E = (S_{COL} - S_{DEL})\, X\, 100 \qquad \text{Equation 5.3}$$

where E = efficiency
S_{COL} = solvent collected
S_{DEL} = solvent delivered

On average, the efficiency for samples that had been collected using EDTA was 99.9%, using 2% HNO_3 was 100.3% and DI H_2O achieved 95.9% efficiency. The 100.3% average using a delivery solvent of 2% HNO_3 suggests that extra solvent has been picked up from previous samples. A closer look at the breakdown shows that the blank efficiency was 102.3%. This may be due to traces of the sampling solvent from previous testing, in possibly the tubes of the LSP, following cleaning of the device prior to collecting the blank sample. Also, the first sample (B) collected using the sampling solvent of DI H_2O showed an efficiency of 78.2%, very low in comparison to all other recoveries. For this to occur, some of the solvent delivered would not have been transferred to the collection vial via the target sampling site. It may have been possible that some of the sampling solvent could have been left in the transfer tubes or if a seal had not been retained at the sampling base of the LSP, the sample could have leaked out around the target surface and lost. Previously, some solvent had been observed to go into the collection vial via one needle but would form a drop at the end of the needle, a drop big enough to then touch the needle that led to the peristaltic pump outlet. This would then suck up the drop over this needle and the collected sample would go through the peristaltic pump tubing to waste.

The observed differences in collection efficiency were not due to different uptake from the delivery vial.

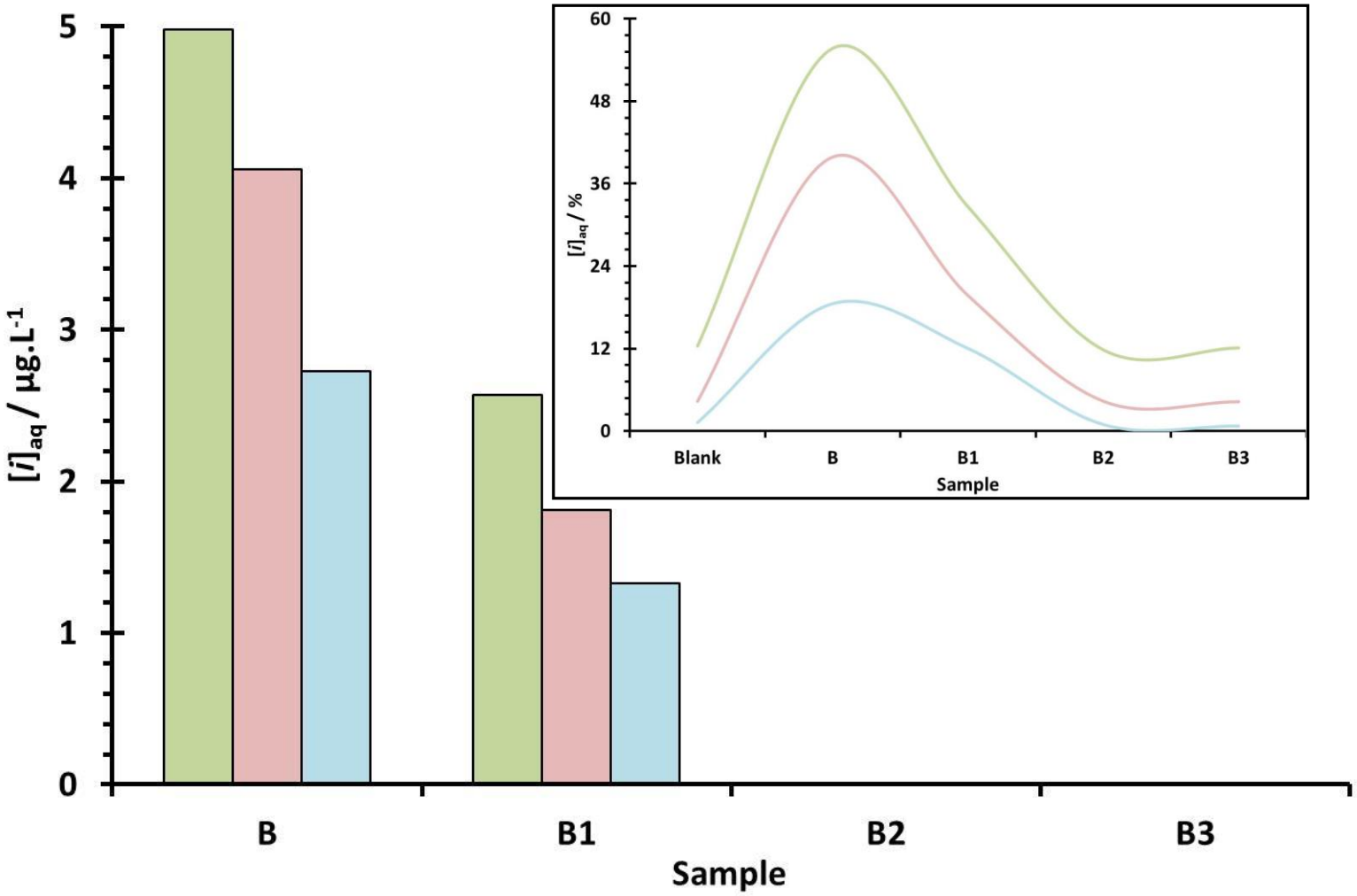


Figure 5.8. Mn concentration (ng.mL^{-1}) for the comparison of the three different sample solutions; 2% HNO_3 (green); EDTA (pink); and DI H_2O (blue). Insert: Recovery (%) of the three different sample solutions. B = reference sample, B1 = wash sample 1, B2 = wash sample 2 and B3 = wash sample 3

H_0 was rejected, as a statistically significant difference was found in the recovery of a Mn sample from a nitrile glove using the LSP, between sampling solvents (H_0: S_S *(x)* ≠ S_S *(y)*). A pre-selected sample was extracted using three sampling solvents with highest first recoveries observed for 2% HNO_3 (55.7%) followed by EDTA (39.9%) and finally DI H_2O (18.6% recovery). Although samples collected using the sampling solvent 2% HNO_3 produced the best recoveries, this solvent is not suitable for use on human skin. However, using EDTA as the sampling solvent produced acceptable recoveries and is better suited to collecting samples from the surface of human skin. EDTA is a good chelating agent that can extract metals from the skin surface better than DI H_2O and is less hazardous than 2% HNO_3.

5.6 Non-invasive fast skin profiling for elements from the volar and plantar

The aim of this study was to investigate the differences in elemental profiles between the skin volar (AE) and skin plantar (BF), using the LSP. Samples were collected from pre-determined sites from two sampling areas of human skin, using EDTA as the LSP sampling solvent. All samples were collected and analysed by the author and Dr Corrinne Burns (personal communication). H_0 of the experiment was there was no differences in the elemental profiles between human volar (AE) and plantar (BF) skin sites i.e. H_0: *volar* $(AE)_E$ = *plantar* $(BF)_E$.

This experiment follows a similar workflow as that outlined in Figure 5.4. One LSP was used to collect three samples, using EDTA as the sampling solvent, per participant; (in order) 1 x LSP pen blank, 1 x volar (AE) site and 1 x plantar (BF) site. A total of 60 vials and 120 vial caps were used for this study. Ten male and/or female volunteers aged between 18 and 35 years participated in the study. Participants were asked not to shower or use any deodorants or products on their skin or hair for fifteen hours prior to skin sampling. Only one participant was sampled at any one time and each participant was required for sampling for only one day. For each participant 6 vials and 12 vial caps were used, 2 vials and 4 caps each for collecting the LSP pen blank and sampling both the volar (AE) and dorsal (TF) skin sites. 10 complete sets of sampled skin were obtained and analysed over 3 days by ICP-MS.

5.6.1 Experimental Outline

1 LSP was used to collect a total of 30 samples, 3 from each participant. The skin sampling areas of the volar (AE) and plantar (BF) can be observed in Figure 5.2.

5.6.1.1 Sampling the volar

The volar (AE) sample site was cleaned with DI H_2O using a clean pad of cotton, prior to sample initiation. The volar (AE) was sampled first, with 1.5 ml EDTA, using the LSP.

5.6.1.2 Sampling the plantar

Following this, the LSP was cleaned and a further LSP pen blank sample was collected. Participants were then asked to remove the sock from their right foot and place their dorsal (TF) of the bare foot flat on a chair. The plantar (BF) sample site was cleaned with DI H_2O using a new cotton pad prior to collecting the plantar (BF) site sample.

Following post collection weighing of the sample vials, all samples were stored in a freezer at -80°C until the morning of analysis.

5.6.1.3 Sample Analysis

Samples were allowed to thaw at room temperature for 1 hour after which they were homogenised using a vortex mixer. For each sample 1 ml of the sampled solvent was diluted with 4 ml 2% HNO_3 in a pre-cleaned (with 2% HNO_3) polypropylene tube and labelled ready for analysis.

Elemental detection of twenty six elements was performed using ICP-MS (Chapter 1 section 1.3.5). A 2% HNO_3 solvent blank was bracketed in the analysis sequence every two samples to assess the baseline elemental levels of the instrument. Also, all samples were analysed in triplicate to obtain more reliable results. During data analysis the dilution factor for all samples was taken into account after which LSP pen blanks were subtracted from the relevant samples. Volar (AE) and plantar (BF) samples were grouped in order to perform ANOVA and then a paired t-test.

5.6.2 Results and Discussion

A total of 32 samples were analysed that included 10 samples from the plantar (BF), 10 samples from the volar (AE), 10 LSP pen blank samples, 1 EDTA solvent sample and 1 EDTA vial blank sample. From the 26 elements that were determined, 8 were detected in the two sample sites (Table 5.6).

As shown in Table 5.6 elemental concentrations from the volar (AE) followed the order (highest first) Si > S > K > Al > Fe > P > Zn > Ni, and a similar trend was seen for the plantar (BF) except that the order of S and K was reversed. In both cases, concentrations ranged from about 2000 $\mu g.mL^{-1}$ for Si to about 7 $\mu g.mL^{-1}$ for Ni, individual variations being indicated by the standard deviations. The data is plotted in Figure 5.9.

Table 5.6. Mean elemental concentrations (ng.mL^{-1}) and (in brackets) standard deviations (n=10) for the elemental profiling of the volar (AE) and plantar (BF) using the LSP. Probability scores of paired t-test (T (P)) to test significant differences between skin sites (n = 10) also shown

Element	Volar	Plantar (BF)	T (P)
Si	1701 (42.5)	2161 (54.0)	0.12
S	626 (15.7)	358 (8.95)	0.22
K	279 (6.98)	736 (18.4)	0.14
Al	94 (2.35)	103 (2.58)	0.60
Fe	74 (1.85)	41 (1.03)	0.08
P	24 (0.60)	22 (0.55)	0.82
Zn	21 (0.53)	15 (0.38)	0.31
Ni	6.2 (0.16)	7.5 (0.19)	0.47

ANOVA was carried out on the data set to determine whether there was any difference in variance between the two skin sample sites (Appendix Table 8.12). There was no statistical evidence of significant difference in the variation between the two skin sample sites as the calculated F value, 1.11, was much lower than the critical F value, 3.91. This is confirmed by the results of a paired t test that was performed to identify significant differences in elemental recoveries from the two skin sample sites. All T (P) values are above 0.05 indicating there is no statistically significant evidence of differences in elemental recoveries between the two skin sample sites (Table 5.6). As expected, there are differences observed in variation between the amounts of different elements recovered from the sample sites as the calculated F value (40.11) is much bigger than the critical F value (2.07).

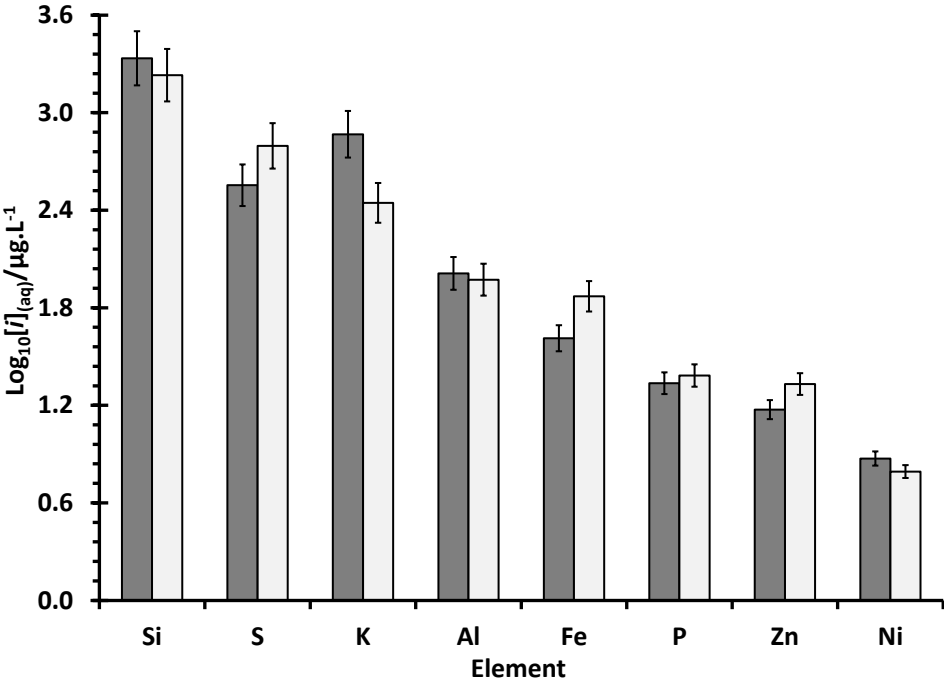


Figure 5.9. Mean concentrations of 10 participants for plantar (BF) (50% grey) and volar (AE) (5% grey) skin sites of the eight recovered elements using the LSP. The error bars designate standard error of the mean (95% confidence)

H_0 was accepted as no difference in elemental profiles between human volar (AE) and plantar (BF) skin sites (*H_0: volar $(AE)_E$ = plantar $(BF)_E$*) was discovered. Although 8 elements were extracted from the two sample sites, there was no statistically significant evidence of differences in the elemental content between the two sample sites.

High Si levels were found in both the volar (AE) and plantar (BF) skin sample sites. There is a possibility that this was the result of Si leaching from the LSP vials and transport tubing. As a result, an updated LSP cleaning method should be implemented removing any impurities from future samples. The LSP should be pre cleaned and dried in a vacuum oven prior to collecting samples to reduce any elemental carry over from previous samples. Further to this, a new peristaltic pump should be used in order to prevent sample loss via the peristaltic pump tubing, something that was observed during sample collection.

The skin sample sites should not be treated with deionised water prior to sample collection as this procedure may remove any elemental traces that may be of interest. Instead samples should be collected from skin sample sites without any pre-treatment.

5.7 Non-invasive fast skin profiling for elements from the axilla, dorsal, plantar and volar

The aim of this study was to investigate the differences in elemental profiles obtained between the four skin sites on the human body using the LSP. Samples were collected from the axilla (AP), dorsal (TF), plantar (BF) and volar (AE) using an EDTA sampling solvent. The experiment was conducted by Dr Svetlana Riazanskaia (personal communication) and all samples were collected and analysed by the author and Dr Corrinne Burns (personal communication). H_0: there was no difference in elemental profiles, obtained using the LSP, between human skin axilla (AP), dorsal (TF), plantar (BF) and volar (AE) sites i.e. *H_0: axilla $(AP)_E$ = dorsal $(TF)_E$ = plantar $(BF)_E$ = volar $(AE)_E$*.

This experiment follows a similar workflow as that outlined in Figure 5.4. A total of 10 LSPs were used with each LSP collecting 8 samples, using EDTA as the sampling solvent, per participant; (in order) 1 x LSP pen blank axilla (AP) site, 1 x axilla (AP) site, 1 x LSP pen blank dorsal (TF) site, 1 x dorsal (TF) site, 1 x LSP pen blank plantar (BF) site, 1 x plantar (BF) site, 1 x LSP pen blank volar (AE) site, 1 x volar (AE) site. A total of 160 vials and 320 vial caps were used for this study. For each participant 16 vials and 32 vial caps were used, 8 vials and 16 caps each for collecting the LSP pen blank and sampling all four sample skin sites. 10 complete sets of sampled skin were obtained over 2 days. Sample analysis commenced 5 days after collection by ICP-MS over a 27 day period.

5.7.1 Experiment Outline

Ten (5 male and 5 female) volunteers aged between 18 and 59 years participated in the study. Participants were recruited by internal advertising within Unilever PLC, Bebington. Participants refrained from using perfumed soap, shampoo and deodorant for three weeks prior to the start of skin sampling. Participants were provided with unperfumed cosmetics and were allowed to use these as frequently as necessary. Participants were provided with *Simple* unfragranced soap to use for showering for a week prior to sample collection and were asked not to wash in the twenty-four hours preceding skin sampling. They were also provided with pre-cleaned cotton T-shirts and socks, by Unilever Plc, to wear during this time. Participants were required for only one visit. All female participants were sampled on the first day and all male participants were sampled on the following day.

Assistance for pre-sample preparation and sample collection was given by Dr. Corrinne Burns.

Figure 5.10. Participant information summary for the elemental profiling of the axilla (AP), dorsal (TF), plantar (BF) and volar (AE)

Sample Date	Sample Day	Participant ID	Gender	Sample site				No. of samples	Sample storage
				Axilla	Dorsal	Plantar	Volar		
29/09/2011	1	UP1	Female	Right	Left	Left	Left	8	-80°C
29/09/2011	1	UP2	Female	Left	Right	Right	Right	8	-80°C
29/09/2011	1	UP3	Female	Right	Left	Left	Left	8	-80°C
29/09/2011	1	UP4	Female	Left	Right	Right	Right	8	-80°C
29/09/2011	1	UP5	Female	Right	Left	Left	Left	8	-80°C
30/09/2011	2	UP6	Male	Right	Left	Left	Left	8	-80°C
30/09/2011	2	UP7	Male	Left	Right	Right	Right	8	-80°C
30/09/2011	2	UP8	Male	Left	Right	Right	Right	8	-80°C
30/09/2011	2	UP9	Male	Right	Left	Left	Left	8	-80°C
30/09/2011	2	UP10	Male	Left	Right	Right	Right	8	-80°C

18 LSP's were used to collect a total of 80 elemental samples, 8 from each participant. Participants were asked to lie on their back, on a portable masseuse table, during sample collection. Samples were collected in the following order; axilla (AP), dorsal (TF), plantar (BF) and volar (AE). Participants were asked to remove socks on the selected foot for sampling prior to collecting the dorsal (TF) and plantar (BF) site samples.

Throughout the study, alternate sides of the body were sampled between participants to randomise sample sites and thus avoid bias. For example, if the right axilla (AP) was sampled for the first participant, then the left axilla (AP) would be sampled for the second participant. The skin sampling areas of the axilla (AP), dorsal (TF), plantar (BF) and volar (AE) can be observed in Figure 5.2.

5.7.1.1 Sampling the axilla

A LSP pen blank was initially collected prior to collecting the first sample on the axilla (AP) site (labelled AP Blank). The t-shirt sleeve was lifted to the shoulder of the participant on the selected axilla (AP) sample side. Participants lay back on the masseuse table with the selected arm raised by their ear so the axilla (AP) sampling site was exposed and parallel to the ceiling. One sample was collected from the axilla (AP) site using the LSP with EDTA as the sampling solvent.

5.7.1.2 Sampling the dorsal

Following sample collection from the axilla (AP) site, the LSP was then cleaned. The second LSP pen blank was collected (labelled TF pen blank). Participants then sat up at the end of the masseuse table and removed the sock on the foot selected for sampling, ensuring the foot remained suspended and did not come into contact with the table or floor. With the dorsal (TF) parallel to the ceiling, one sample was collected from the dorsal (TF) using the LSP with EDTA as the sampling solvent.

5.7.1.3 Sampling the plantar

Next, the LSP was then cleaned again. The third LSP pen blank was collected (labelled BF pen blank). Participants were asked to then lie on their stomach ensuring the foot remained suspended and did not come into contact with the table or floor. With the plantar (BF) parallel to the ceiling, one sample was collected from the plantar (BF) using the LSP with EDTA as the sampling solvent.

5.7.1.4 Sampling the volar

Finally, the LSP was then cleaned. The fourth and final LSP pen blank was collected (labelled AE pen blank). Participants were asked to sit on the edge of the masseuse table with the selected sampling arm outstretched so that the volar (AE) sampling site was parallel to the ceiling. One sample was collected form the volar (AE) using the LSP with EDTA as the sampling solvent.

5.7.1.5 Field Blank Collection

One field blank was collected on each of the two days during participant sampling. When the first participant arrived, the cap of one vial containing 1.5 ml EDTA (sampling solvent) was unscrewed and removed from the top of the vial. This was then exposed to the environment of the sampling room until the last sample was collected from the final participant on that

given day. The cap was then replaced and the vial was placed in storage, with all other vials, after which samples were stored in a freezer at -80°C until the morning of analysis.

5.7.1.6 Sample Analysis

Samples were prepared for analysis as detailed in section 5.6.1.3. Elemental detection of twenty three elements was performed using ICP-MS (Chapter 1 section 1.3.5).

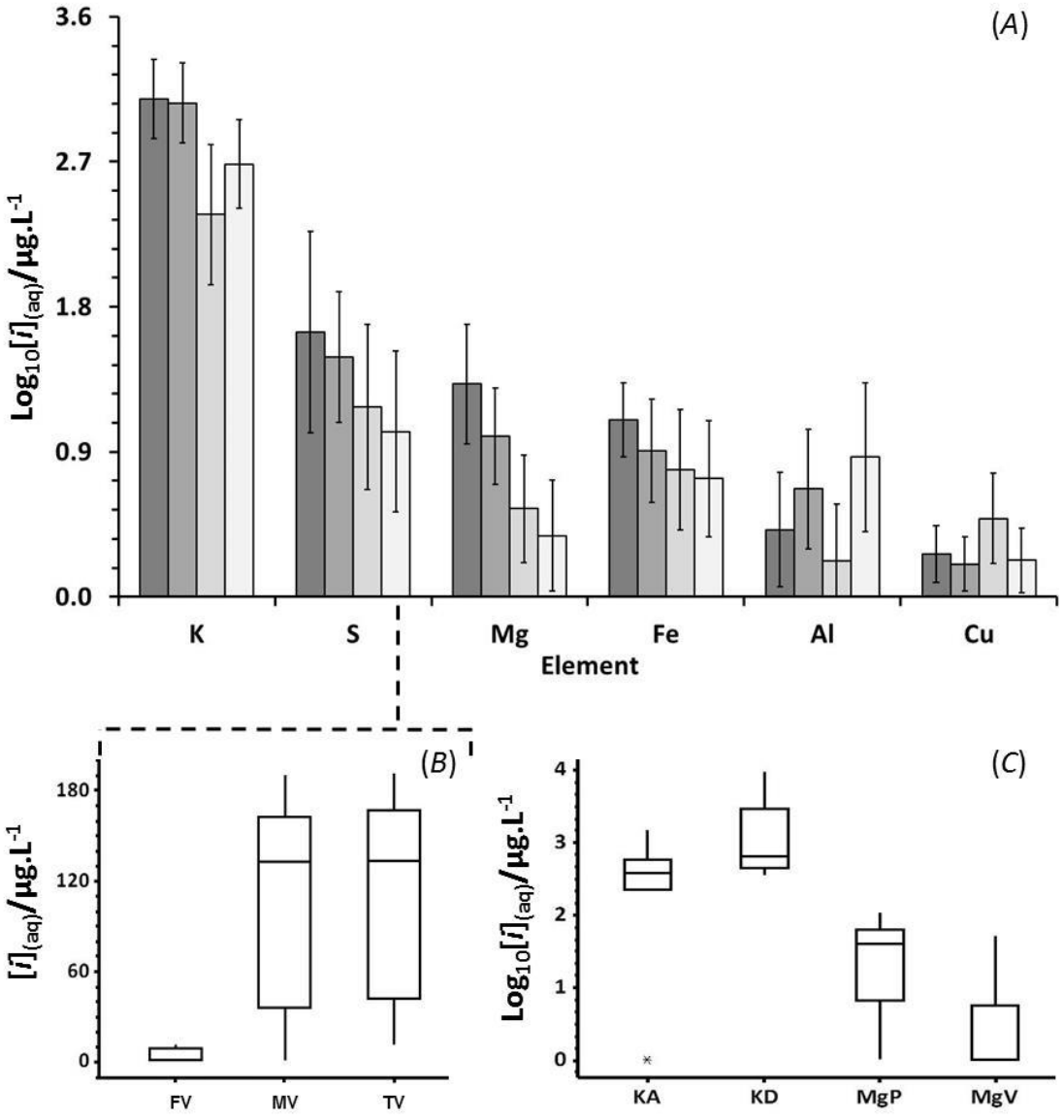


Figure 5.11. (A) Mean concentrations of 10 participants for plantar (BF) (50% grey), dorsal (TF) (35% grey), axilla (AP) (15% grey) and volar (AE) (5% grey) skin sites of the six recovered elements using the LSP. The error bars designate 1 standard deviation of the mean. (B) Box-whisker plot of female volar (FV), male volar (MV) and combined volar (TV) results for S. (C) Box-whisker plot for the K axilla (AP) (KA), K dorsal (TF) (KD) Mg plantar (BF) (MgP) and Mg volar (AE) (MgV) skin sites

Table 5.7. Element concentrations (µg/L) and standard deviations (n = 5) from female (F) and male (M) skin sites sampled using the LSP. Probability (P) of t-test testing significance between genders for each sample site where values of P < 0.05 shows evidence of significant difference (at 95%)

Element	Axilla			Dorsal			Plantar			Volar		
	F	M	P	F	M	P	F	M	P	F	M	P
K	600 (560.0)	335 (129.6)	0.68	2768 (3966.4)	1614 (1537.4)	0.87	1178 (745.8)	3096 (4214.0)	0.29	495 (403.0)	1221 (1119.3)	0.37
S	55 (75.5)	61 (36.0)	0.35	37 (36.3)	93 (42.4)	0.13	1237 (2675.8)	133 (84.0)	0.78	4 (5.2)	107 (73.1)	**0.03**
Mg	18 (36.5)	5 (6.0)	0.92	23 (17.3)	11 (9.3)	0.46	24 (18.1)	61 (40.4)	0.50	8 (17.2)	10 (23.4)	0.88
Fe	9 (12.7)	27 (38.3)	0.33	14 (15.3)	15 (10.3)	0.66	23 (10.7)	12 (11.0)	0.11	18 (38.1)	14 (8.1)	0.29
Al	0 (0)	34 (76.5)	0.35	3 (7.6)	24 (24.3)	0.07	0 (0.0)	21 (30.5)	0.06	10 (22.8)	48 (33.7)	0.07
Cu	2 (3.2)	10 (14.5)	0.33	1 (2.2)	2 (2.7)	0.57	2 (3.3)	2 (2.3)	0.87	3 (5.7)	2 (2.4)	0.97

Table 5.8. Element concentrations (µg/L) and standard deviations (n = 5) from right (R) and left (L) skin sites sampled using the LSP. Probability (P) of t-test testing significance between sample side for each sample site where values of P < 0.05 shows evidence of significant difference (at 95%)

Element	Axilla			Dorsal			Plantar			Volar		
	R	L	P	R	L	P	R	L	P	R	L	P
K	392 (278)	543 (532)	0.43	3371 (3814)	1011 (951)	0.32	1279 (559)	2995 (4296)	0.94	806 (781)	910 (1062)	0.97
S	41 (63)	75 (49)	0.25	82 (31)	48 (59)	0.16	106 (71)	1264 (2662)	0.93	42 (61)	69 (88)	0.50
Mg	0 (0)	24 (34)	**0.00**	14 (9)	20 (20)	1.00	48 (30)	37 (43)	0.71	0 (0)	19 (25)	0.08
Fe	7 (11)	29 (38)	0.23	8 (11)	21 (10)	0.07	18 (7)	16 (16)	0.38	8 (10)	24 (35)	0.35
Al	0 (0)	34 (76)	0.35	12 (14)	16 (27)	0.80	21 (30)	0 (0)	0.06	20 (32)	38 (36)	0.50
Cu	3 (5)	9 (15)	0.50	2 (2)	1 (3)	0.63	1 (2)	3 (3)	0.18	1 (1)	4 (6)	0.29

5.7.2 Results and Discussion

A total of 84 samples were analysed which included 10 samples each from the axilla (AP), dorsal (TF), plantar (BF) and volar (AE), 40 LSP blank samples and 4 solvent blank samples. From the 23 elements that were measured, 6 were detected in all four sample sites. While all elements detected were observed in each sample site, the highest concentrations were observed in the plantar (BF) sample site followed by the dorsal (TF), axilla (AP) then volar (AE). K, S, Mg and Fe were discovered in large quantities in the plantar (BF) sample site with concentrations of 2137, 685, 43 and 17 ng.mL^{-1}, respectively. Smaller amount of Al and Cu were also found in all sample sites where the largest concentration of Al found in the volar (AE) and the largest concentration of Cu found in the axilla (AP), were concentrations at 29 and 6 ng.mL^{-1}, respectively (Table 5.9).

Table 5.9. Mean element concentrations (ng.mL^{-1}) and (in brackets) standard deviations (n=10) for the elemental profiling of the axilla (AP), dorsal (TF), plantar (BF) and volar (AE) using the LSP

Element	Plantar	Dorsal	Axilla	Volar
K	2137 (53.4)	2191 (54.8)	468 (11.7)	858 (21.5)
S	685 (17.1)	65 (1.63)	58 (1.45)	55 (1.38)
Mg	43 (1.08)	17 (0.43)	12 (0.30)	9 (0.23)
Fe	17 (0.43)	15 (0.38)	18 (0.45)	16 (0.40)
Al	11 (0.28)	14 (0.35)	17 (0.43)	29 (0.73)
Cu	2 (0.05)	1 (0.15)	6 (0.15)	2 (0.05)

Initially, ANOVA (two-factor with replication, n = 10, P = 0.05) was conducted (Appendix Table 8.14) to observe if any differences between the four sample sites was present. The F value (3.75) obtained was larger than the critical F value (2.65) between sample sites, suggesting there is evidence of differences in the elemental concentrations present. Also, a calculated F value of 62.19 was obtained, much bigger than the critical F value of 2.26.

Table 5.10 shows the probability values (P) of a paired t-test between the four skin sites. Evidence of significant difference (95%) in elemental concentration was present for K between the axilla (AP) and dorsal (TF) sample sites and also a difference in Mg between the plantar (BF) and volar (AE) sample sites. Mg plantar (BF) concentrations averaged 43 µg.mL^{-1} whereas those found in the volar (AE) averaged only 9 µg.mL^{-1}. K axilla (AP) concentrations averaged 468 µg/ml. In contrast, the dorsal (TF) site K concentration averaged 2191 µg.mL^{-1}.

Evidence of gender differences for S in the volar (AE) were observed using a t-test (95%) (Figure 5.11 (*B*)). The concentration in males was found to be significantly higher than in females with an average S concentration of 107 µg.ml^{-1} observed in male volar (AE) whereas only 4 µg.ml^{-1} was observed in female volar (AE). Statistically significant differences were

also found for Mg between the axilla (AP) sides. An average of 24 µg.ml^{-1} was determined in the left axilla (AP) of participants whereas the amount of Mg in the right side was below the limit of detection.

Table 5.10. Probability scores of paired t-test to test significant differences between skin sites (n = 10) sampled using the LSP

Element	Axilla & Dorsal	Axilla & Plantar	Axilla & Volar	Dorsal & Plantar	Dorsal & Volar	Plantar & Volar
K	0.03	0.06	0.35	0.93	0.16	0.10
S	0.29	0.44	0.73	0.74	0.22	0.19
Mg	0.14	0.05	0.66	0.27	0.07	0.04
Fe	0.74	0.20	0.85	0.52	0.51	0.23
Al	0.13	0.50	0.05	0.33	0.53	0.25
Cu	0.29	0.35	0.16	0.70	0.91	0.84

H_0 was rejected as differences in elemental profiles between human skin axilla (AP), dorsal (TF), plantar (BF) and volar (AE) sites were found (H_0: *axilla (AP)*$_E \neq$ *dorsal (TF)*$_E \neq$ *plantar (BF)*$_E \neq$ *volar (AE)*$_E$.). Across the four sampling sites, 6 elements were detected where K was found to be the highest in each of the four sample sites, especially in the axilla (AP) and volar (AE) sites, when compared to the other elements. Other elements detected included S, Mg, Fe, Al and a trace amount of Cu. Statistically significant differences were demonstrated for K between axilla (AP) and dorsal (TF) sampling sites, Mg between plantar (BF) and volar (AE) sites, S from the volar (AE) sample site between male and females, and Mg in the axilla (AP) between the right and left sample site.

5.8 Non-invasive fast profiling of the dorsal and plantar for semi-volatile organic compounds and non-volatile organic compounds

The objective of this study was to identify and investigate the differences in semi-volatile organic compounds (semi-VOCs), volatile organic compounds (VOC) and small molecule profiles obtained between the dorsal (TF) and plantar (BF) skin sites on the human body using the LSP. Samples were collected from the dorsal (TF) and plantar (BF) using 50% v/v $EtOH_{(aq)}$ sampling solvent. All samples were collected by the author and analysed by the author with assistance from Dr. James Reynolds (personal communication). All data was processed by the author. H_0: there was no difference in profiles, obtained using the LSP, between human skin dorsal (TF) and plantar (BF) sites i.e. H_0: *dorsal (TF)*$_V$ = *plantar (BF)*$_V$.

The LSP was evaluated as a non-invasive method to obtain small molecules in human skin. Extracted small molecules were collected from the dorsal (TF) and plantar (BF) from a sample solution containing 1ml $EtOH_{(aq)}$ using the LSP.

This experiment follows a similar workflow as that outlined in Figure 5.4. In the pre-sampling phase of the study, all eighteen LSP's were cleaned with selected solvents and samples were obtained using 50% v/v EtOH as the sampling solvent and analysed by ESI-MS. Acceptable (low) baseline levels were observed for all LSP's. All LSP's were re-cleaned ready for sample collection from participant skin. Socks, pre-cleaned in non-fragranced washing powder, and a non-fragranced body soap bar (Simple) were given to the participants prior to the morning of sampling. Collection and delivery vials were weighed before and after sample collection. 50% v/v EtOH was used as the sampling solvent and 1.5 ml was added to delivery vials and attached to the LSP's ready for sample collection. Pre weighed and clean collection vials were also attached to the LSP.

Ten (5 male and 5 female) volunteers aged between 18 and 35 years participated in the study. Participants were provided with *Simple* non fragranced soap to shower with the night prior to sample collection and were asked not to wash the morning of sampling. Participants were also provided with pre-cleaned cotton socks to wear during this time and were asked to refrain from using deodorants or products for the skin or hair and not consume any food or drink (other than unflavoured water) on the morning of sampling. Nine complete sets of sampled skin were obtained over a period of thirteen days with participants required for one visit. Males and females were alternatively sampled.

In the sample collection phase of the study, two LSP's were used to collect skin samples from each participant, one LSP to collect from the dorsal (TF) and one from the plantar (BF). LSP pen blanks were collected from each LSP before the participant arrived. Samples were collected using 50% v/v EtOH. A total of eighteen LSP's were used with each LSP collecting one sample, using EtOH as the sampling solvent, per participants; (in order) 1 x LSP pen blank dorsal (TF) site (LSP pen 1), 1 x LSP pen blank plantar (BF) site (LSP pen 2), 1 x LSP dorsal (TF) site (LSP pen 1), 1 x plantar (BF) site (LSP pen 2). A total of 92 vials and 130 vial caps were used for this study. For each participant, 9 vials and 13 vial caps were used, 8 vials and 12 caps each for collecting the LSP pen blanks and 1 vial and 1 cap to collect a field blank during sample collection. Samples were stored at -80°C until day of analysis.

5.8.1 Experiment Outline

18 LSP's were used to collect a total of 40 samples, 4 from each participant. Also collected from each participant was a field blank sample. Participants were asked to sit on a chair during sample collection of the dorsal (TF) and place their knee on a chair for the collection of the plantar (BF) sample. LSP pen blank samples were collected from each LSP prior to participant arrival. One field blank sample was collected while participant was in the

sampling room. Dorsal (TF) samples were always collected first followed by the plantar (BF) samples. Samples were collected, using the method outlined in Chapter 5 section 5.3. Participants were asked to remove socks on the selected foot for sampling prior to collecting samples. Immediately after each site was sampled, the vial of the collected sample was recapped with a new, clean screw cap with an un-pierced rubber septum. Once all samples were collected for each participant, the samples were refrigerated at -80°C until analysis.

Each participant was required for a single 30 minute session in order to fill out the relevant documentation as well as complete the sample collection process. Once samples were collected, the socks that the participants had been wearing were wrapped in foil and placed in a sealed secondary container. Participants were asked to bring a spare pair of socks with them to wear following the sample collection session. Once samples were collected, the participant was free to leave.

In the sample analysis stage of the experiment, sample were allowed to thaw at room temperature on the morning of analysis and then analysed by ESI-MS in firstly negative and then positive ion mode. Raw data was obtained by peak profiling after which MVA was conducted and then descriptive statistics was performed. Accurate mass determination and possible ion identity was conducted nESI-MS.

Assistance for sample collection and ESI-MS and nESI-MS analysis was given by Dr. James Reynolds. ESI-MS and nESI-MS conditions can be found in Chapter 1 sections 1.4 and 1.5, respectively.

The skin sampling areas of the dorsal (TF) and plantar (BF) can be observed in Figure 5.2 (*B*) & (*C*).

5.8.1.1 Protection of dorsal and plantar sample sites

The purpose of protecting the sample site was to avoid any contamination of the feet. 20 white cotton socks (10 with a grey marking to be used for male participants and 10 with a pink marking to be used for female participants) were washed in a washing machine (Bosch Exxcel 1400 Express) at 30°C for 62 minutes with non-fragranced washing powder (Persil). Following the completion of the wash cycle all socks were then placed into a tumble dryer (Zanussi Electrolux Condenser Dryer, TCE 776 W) for 75 minutes. Once the cycle was completed, the socks were paired, wrapped in foil (shiny side outwards for males and dull side outwards for females) and placed into a sealed secondary container.

Two days prior to the sample collection date, participants were provided with a participant information sheet, clean socks and non-fragrance bar of *SIMPLE* soap. The night before sample collection participants washed both of their feet using the provided soap and did not use any other skincare products until sample collection. Feet were covered immediately after being washed with the provided sock and these were not removed until the feet were sampled the following morning. While the socks were on the feet, participants were told not to consume any food or drink (except water).

5.8.1.2 LSP pre sample cleaning

All LSP's were cleaned and dried using the sample procedure outlined in Chapter 1 section 5.3.2. Following this first clean, LSP pen blanks were collected using the sampling solvent EtOH. 18 samples were analysed by ESI-MS in negative ion mode and blank levels were viewed in MassLynx and deemed low enough to be acceptable.

All eighteen* LSP's were cleaned and dried again, using the outlined method, ready for sample collection.

*An extra cycle of cleaning and drying was carried out for two LSP's once participant samples had been collected using them due to the need to have twenty clean LSP's.

5.8.1.3 Foot Selection

Within each gender, an alternative foot was selected to be sampled. For example, the first male participant was chosen to have the left foot sampled thus the second and third male participants would have the right and left foot sampled, respectively. The same selection process was applied to the female participants where the first female participant's right foot was sampled first. This resulted in 3 right foot samples and 2 left foot samples selected for male participants and 3 left foot samples and 2 right foot samples being selected for female participants. Consequently, 5 right foot samples and 5 left foot samples were taken in total (supplementary information Table SI.5.3).

5.8.1.4 Sampling the dorsal

The participant was asked to sit on a chair and remove their shoes and socks (socks were immediately wrapped in foil and placed in a sealed container) on the selected foot, resting the heel of the foot on a piece of paper towel on the floor ensuring that the plantar (BF) of the foot did not come into contact with the floor and the dorsal (TF) was parallel to the ceiling (or as close to parallel as possible without the dorsal (TF) of the foot touching the floor). One sample was collected from the dorsal (TF) site using LSP 1 with EtOH as the

sampling solvent. The collection vial cap was then replaced with a new screw cap with no piercings from the LSP sample needles. LSP 1 was then re-racked.

5.8.1.5 Sampling the plantar

Following sample collection from the dorsal (TF) site, the participant carefully turned around and placed their knee on the chair, holding on to the back of the chair to balance, ensuring the plantar (BF) of the sampling foot did not come in contact with the floor. The foot was raised so the plantar (BF) site was now parallel to the ceiling. One sample was collected from the plantar (BF) site using LSP 2 with EtOH as the sampling solvent. The collection vial cap was then replaced with a new screw cap with no piercings from the LSP sample needles. LSP 2 was then re-racked.

5.8.1.6 Field Blank Collection

One field blank was collected for each participant. When the first participant arrived, the cap of one vial containing 2 ml EtOH (same as the sampling solvent) was unscrewed and removed from the top of the vial. This was then exposed to the environment of the sampling room until the participant left the sample room. The cap was then replaced and the vial was placed in storage with all other vials.

Samples were stored in a freezer at -80°C until the morning of analysis.

5.8.1.7 Sample Analysis

All 51 (5 per participant and 1 EtOH sampling solvent blank) samples were analysed on the same day. The storage duration of the sample vials was between 22-35 days for ESI-MS negative mode analysis, and between 138-151 days for ESI-MS positive mode analysis. On the day of analysis samples were allowed to thaw at room temperature for 1 hour prior to commencing sample analysis. All samples were analysed by ESI-MS (Chapter 1 section 1.4). Four plantar (BF) samples from randomly selected participants (UF1, UF4, UF5 and UF8) were analysed by nESI-MS in an attempt to determine the mass accuracy, as well as the identity, of ions (Chapter 1.5).

5.8.2 Results and Discussion

A total of 51 samples were analysed that included 10 dorsal (TF) samples, 10 plantar (BF) samples, 10 field blank samples, 20 LSP blank samples and 1 solvent sample by ESI-MS.

H_0 was rejected as there were differences in profiles between human dorsal (TF) and plantar (BF) sample sites analysed by ESI-MS (H_0: *dorsal (TF)$_Y$ ≠ plantar (BF)$_Y$*). A total of 96 ions were detected in samples analysed by ESI-MS in positive or negative ion mode (data processing procedures for these samples are outlined in Chapter 1 section 1.6) and 60 ions showed significant differences (95%) between the two skin sample sites. A PCA model based on the ions significant to each sample site, obtained by supervised modelling, predicted dorsal (TF) versus skin sites with 100% sensitivity and selectivity using negative ion mode sample data and 90% sensitivity and 100% selectivity using positive ion mode sample data.

5.8.2.1 ESI-MS Negative ion mode

Over 54400 features were identified between m/z 50-4000 and over 20100 between m/z 50-200 in negative ion mode (Figure 1.9). Many were exogenous background contaminants.

Multivariate Analysis

34 ions were discovered from profiling data using MassLynx. From the initial 34 ions (Table 5.11), 13 ions were selected from the S-plot (Figure 5.12 (C)) created from the PLS-DA model. These 13 ions were furthest from the centre of the S-plot suggesting possible difference between the dorsal (TF) and the plantar (BF) sample regions. The ions selected were found to have observed m/z of 74.0248, 89.0245, 90.0278, 92.9281, 93.0346, 104.0354, 115.0185, 116.0506, 118.0511, 128.0354, 129.0389, 130.0875 and 137.0358.

Using these thirteen variables, PCA was performed using all twenty observations producing a model with two significant components with a cumulative variation (R^2X) of 98.7% of which 97.5% could be cross validated (Q^2X). Two clusters can be observed in the score scatter plot (Figure 5.12 (A)). Plantar (BF) samples appear to be tightly clustered whereas the dorsal (TF) samples are slightly more spread out. However, one observation, UF2 TF, is positioned closer to the plantar (BF) cluster than any other dorsal (TF) observation.

Data obtained from participant 2 was excluded from the data analysis due to the participant not conforming to the pre-study guidelines outlined in the participant information sheet. During the morning of sampling, prior to sample collection, participant 2 ate breakfast consisting of toast, vegetable salad (carrots, peas, cauliflower) and pork with a glass of orange juice. As a result, data for participant 2 was not used.

Using the thirteen variables and only eighteen observations (participant 2 dorsal (TF) and plantar (BF) data excluded), a model was produced consisting of four significant components with a cumulative variation (R^2X) of 99.9% of which 99.1% could be cross validated (Q^2X). Two clusters can be observed in the score scatter plot (Figure 5.12 (B)). Plantar (BF) samples appear to be tightly clustered whereas the dorsal (TF) samples are slightly more spread out.

The sensitivity of the data producing this model was 100% as all 9 plantar (BF) observations (true positives) were grouped together with no dorsal (TF) observations (false positives) within the group. Also, the specificity was 100% as all nine dorsal (TF) observations (true negatives) were grouped together with no plantar (BF) observations (false negatives) within the group.

The weight contributions of the selected 13 ions from the s-plot where then examined and the biggest ion contributors were removed to highlight the importance of the smaller ions contributors. Removing one ion at a time, a model showing the separation between the dorsal (TF) and plantar (BF) sample site groups was observed with only three ions used in the data set. Ions (variables) with m/z 128.0354, 104.0354 and 93.0346 produced a model with two significant components with eighteen observations with a cumulative variation (R^2X) of 100% of which could be cross validated (Q^2X) and 100% sensitivity and specificity (Figure 5.12 (D)).

Descriptive Statistics

Using the intensity obtained for each ion the differences between the dorsal (TF) and plantar (BF) samples were calculated for all thirty one observed ions from the averages obtained from nine participants (excluding participant 2). ANOVA was performed on the data set to discover whether any differences in variance could be found. At 95%, ANOVA provided a calculated F value of 5.96 between the sample sites (Appendix Table 8.15). This value was much larger than the critical F value of 1.45 thus evidence of significant difference between the sample sites was present. Following this a paired t-test was carried out, on normalised data ($\log_{10}$ of original value) due to the data set not being from a normal distribution, to determine whether the two sample sites were significantly different (Table 5.11). H_0: no difference in intensity obtained from dorsal (TF) and plantar (BF) samples.

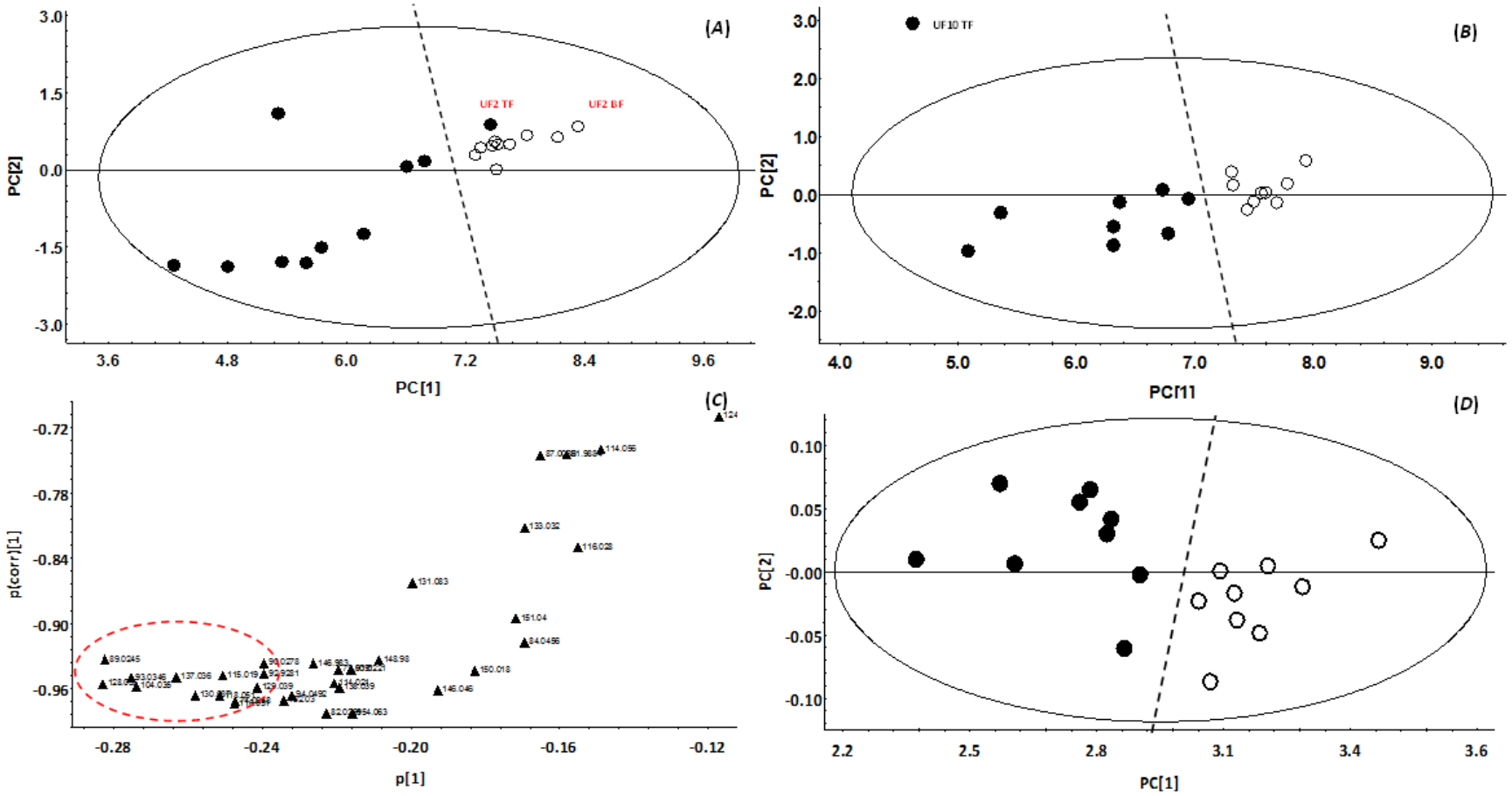


Figure 5.12. Unsupervised PCA of LSP skin sensitive ions identified from PLS-DA on (A) 20 observations and (B) 18 observations, in negative ion mode. 13 ions were used to model the scatter plot from 35 ions identified from PLS-DA. (C) PLS-DA S-plot obtained from 20 observations analysed by ESI-MS in negative ion, R^2X = 96.8%, Q^2X = 89% (D) Unsupervised PCA of LSP skin sensitive ions identified from PLS-DA model on 18 observations in negative ion mode. 3 ions (m/z 93.0346, 104.0354 and 128.0354) were used to model the scatter plot from 34 ions identified from PLS-DA. 100% sensitivity was obtained for the plantar (BF) sample site observations and 100% specificity was obtained for the dorsal (TF) sample site in negative ion mode. R^2X = 100%, Q^2X = 100%. Dots = dorsal (TF) observations, circles = plantar (BF) observation.

The largest difference between the dorsal (TF) and plantar (BF) sample sites was found for the ion m/z 128.0354 with a difference of 51426 counts. Statistically significant evidence of differences between the two sample sites for the ion m/z 128.0354 was discovered at 99.9% using the paired t-test. Other large differences were found from ions with m/z 89, 93, 104 and 137 with intensities obtained of 29209, 27005, 26991 and 10976, respectively. However, it is worth noting that for the ion m/z 89.0245 there was no statistically significant evidence of differences between the two sample sites at 95% whereas for the other four stated ions significant differences were found at 99.9% using a paired t-test. This is attributed to high intensities obtained for this ion from both the dorsal (TF) and plantar (BF) sample sites with the difference found to be smaller than both the plantar (BF) and dorsal (TF) average intensity values.

The ion m/z 89.0245 was present in the air duster (Maplin, Loughborough) used to remove blockages in the sample transfer tubes of the LSP. The smallest difference of intensity between the plantar (BF) and dorsal (TF) sample sites was found for ion m/z 61.9884. For this ion, no statistically significant evidence of differences between the two sample sites was found. Interestingly, for all thirty four ions the intensities obtained for the plantar (BF) was higher than those obtained for the dorsal (TF). From the thirty four ions, using a paired t-test, significant differences were found between the plantar (BF) and dorsal (TF) sample sites for twenty one ions at 95%, eighteen ions at 99% and thirteen ions at 99.9% (Table 5.11).

An F-test followed by a T-test was also carried out in order to investigate whether gender differences existed (5 males, 4 females). Statistical evidence of a significant difference between male and female plantar (BF) samples at 95% was found for only one ion, m/z 129.0389 (Appendix Table 8.16). For this ion, the average intensity obtained for male plantar (BF) samples was 5686, a value higher than that obtained for female plantar (BF) samples of 3564. There was no statistical evidence of significant differences between male and female dorsal (TF) samples (Appendix Table 8.17).

Table 5.11. Mean (μ), standard deviation (σ) and the difference for 34 ion intensities, of dorsal (TF) and plantar (BF) samples, obtained by ESI-MS n negative ion mode. Results of paired t-test (P_{calc}) and whether the H_0 was accepted (✓) or rejected (✗) are shown. Participant 2 excluded from data set. Also shown are accurate ion mass, empirical formula, relative double bond equivalents (RDB), and mass error (δppm) obtained by nESI-MS in negative ion mode from four participant samples. Ions ordered by intensity difference between plantar (BF) and dorsal (TF) samples

Ion (m/z)	TF (Total)		BF (total)		μ (BF) - μ (TF)	P_{calc}	H_0 accepted ($P_{calc}>P_{crit}$)			Ion (m/z)	Empirical formula	RDB	δppm
	μ	σ (n = 9)	μ	σ (n = 9)			P_{crit} (0.05)	P_{crit} (0.01)	P_{crit} (0.001)	Accurate Mass			
128.0354	13792	7186	65218	28492	51426	0.000	✗	✗	✗	128.0354	$C_5 H_6 O_3 N$	3.5	0.887
89.0245	49582	39468	78791	30771	29209	0.201	✓	✓	✓	89.0245	$C_3 H_5 O_3$	1.5	0.591
93.0346	10462	4138	37467	23309	27005	0.000	✗	✗	✗	93.0346	$C_6 H_5 O$	4.5	0.557
104.0354	6923	4440	33914	14697	26991	0.000	✗	✗	✗	104.0354	$C_3 H_6 O_3 N$	1.5	0.996
137.0358	4901	2128	15878	11849	10976	0.000	✗	✗	✗	137.0358	$C_6 H_5 O_2 N_2$	5.5	1.162
130.0875	1611	908	11402	4887	9791	0.000	✗	✗	✗	130.0875	$C_6 H_{12} O_2 N$	1.5	1.215
118.0511	1256	793	7060	3334	5803	0.000	✗	✗	✗	118.0511	$C_4 H_8 O_3 N$	1.5	1.216
132.0304	208	243	5651	2224	5443	0.001	✗	✗	✗	132.0304	$C_4 H_6 O_4 N$	2.5	1.356
116.0506	827	718	5504	2729	4677	0.000	✗	✗	✗	116.0718	$C_5 H_{10} O_2 N$	1.5	1.104
74.0248	1143	1179	5632	2833	4489	0.001	✗	✗	✓	74.0248	$C_2 H_4 O_2 N$	1.5	0.787
92.9281	1608	1537	5789	4796	4181	0.054	✓	✓	✓	92.9281			
115.0185	3124	3341	7303	2189	4179	0.057	✓	✓	✓	115.0185			
72.0091	351	633	4055	2725	3704	0.001	✗	✗	✗	72.0092	$C_2 H_2 O_2 N$	2.5	1.366
129.0389	1337	1368	4743	1780	3406	0.028	✗	✓	✓	129.0389	$C_6 H_9 O S$	2.5	7.215
94.0492	405	221	2310	1940	1905	0.000	✗	✗	✗	94.0493			
82.0299	143	186	1871	1180	1729	0.001	✗	✗	✓	82.0299	$C_4 H_4 O N$	3.5	1.01
87.0088	1795	3082	3243	4347	1448	0.237	✓	✓	✓	87.0088	$C_3 H_3 O_3$	2.5	0.606
131.0828	812	1384	2246	2402	1435	0.056	✓	✓	✓	131.0828	$C_5 H_{11} O_2 N_2$	1.5	1.442
90.0278	2254	2261	3668	1937	1413	0.150	✓	✓	✓	90.0278			
114.0215	512	745	1471	503	959	0.014	✗	✓	✓	114.0198	$C_4 H_4 O_3 N$	3.5	0.822
154.0625	109	99	1053	829	944	0.002	✗	✗	✓	154.0625	$C_6 H_8 O_2 N_3$	4.5	1.948
146.9833	956	889	1805	1091	849	0.119	✓	✓	✓	147.0454	$C_9 H_7 O_2$	6.5	1.817

138.0392	271	193	1050	935	779	0.000	×	×	×	138.0392	$C_7 H_8 N S$	4.5	6.641
114.0561	247	411	972	1048	726	0.268	✓	✓	✓	114.0562	$C_5 H_8 O_2 N$	2.5	0.861
133.0316	354	612	924	841	570	0.208	✓	✓	✓	133.0316	$C_5 H_9 O_2 S$	1.5	-9.874
116.0278	17	52	525	556	507	0.002	×	×	✓	116.0507	$C_8 H_6 N$	6.5	0.926
146.0461	35	61	513	354	477	0.000	×	×	×	146.0461	$C_5 H_8 O_4 N$	2.5	1.636
151.0403	0	0	471	221	471	0.178	✓	✓	✓	151.0403	$C_8 H_7 O_3$	5.5	1.606
84.0456	3	9	425	541	422	0.000	×	×	×	84.0456	$C_4 H_6 O N$	2.5	0.747
91.0221	443	376	786	292	343	0.093	✓	✓	✓	91.0221	$C_3 H_7 O S$	0.5	-1.416
124.0075	18	53	296	609	279	0.049	×	✓	✓	124.0074	$C_2 H_6 O_3 N S$	0.5	1.153
150.0175	34	49	278	157	245	0.002	×	×	✓	150.174	$C_3 O N_7$	7.5	3.46
148.9803	335	247	568	325	233	0.162	✓	✓	✓	148.9803	$C_6 H O N_2 S$	7.5	-8.1
61.9884	461	465	670	701	210	0.264	✓	✓	✓	61.9884	$O_3 N$	1.5	0.545
					H_0 accepted		13	16	21				
					H_0 rejected		**21**	**18**	**13**				
					Total		34	34	34				

5.8.2.2 ESI-MS Positive ion mode

Over 177200 features were identified between m/z 50-4000 and over 21500 between m/z 50-200 in positive ion mode. Many were exogenous background contaminants.

Multivariate Analysis

61 ions were discovered from profiling data using MassLynx. From the initial 61 ions (Table 5.12), 7 ions were selected from the S-plot (Figure 5.13 (A)) modelled from PLS-DA. These 7 ions were furthest from the centre of the S-plot suggesting possible differences between the dorsal (TF) and the plantar (BF) sample regions. The ions selected were found to have observed m/z 112.9112, 114.9070, 116.0842, 120.0073, 121.0520, 139.0653, 150.0297.

PCA was performed using the seven variables and all twenty observations producing a model with two significant components with a cumulative variation (R^2X) of 81.5% of which 60.8% could be cross validated (Q^2X) (Figure 5.13 (B)). Two clusters can be observed in the score scatter plot (Figure 5.13 (A)). Plantar (BF) samples appear to be tightly clustered whereas the dorsal (TF) samples are slightly more spread out. However, two dorsal (TF) observations, UF2 TF and UF5 TF, are positioned within the plantar (BF) cluster rather than the dorsal (TF) cluster. The model shows 83.3% sensitivity for plantar (BF) observations (true positives) 100% specificity for dorsal (TF) samples (true negatives). Also, observation UF2 BF appears outside the 95% hoteling region. This is further proof that participant 2 should be excluded from the data set due to incompliance prior to sample collection.

Using the seven variables and only eighteen observations (participant 2 dorsal (TF) and plantar (BF) data excluded), the model produced contained two significant components with a cumulative variation (R^2X) of 99% of which 95.5% could be cross validated (Q^2X). Two clusters can be observed in the score scatter plot (Figure 5.13 (C)). Plantar (BF) samples appear to be tightly clustered whereas the dorsal (TF) samples are slightly more spread out. The model shows 90% sensitivity for plantar (BF) observations (true positives) 100% specificity for dorsal (TF) samples (true negatives). Observation dorsal (TF) UF5 is also located away from the dorsal (TF) cluster. By examining the contribution plots for the UF5 dorsal (TF) sample and comparing this observation to all other dorsal (TF) observation, it is apparent that for the UF5 dorsal (TF) sample the weight of all ion contributors is approximately opposite when compared to all other dorsal (TF) observations (Figure 5.14).

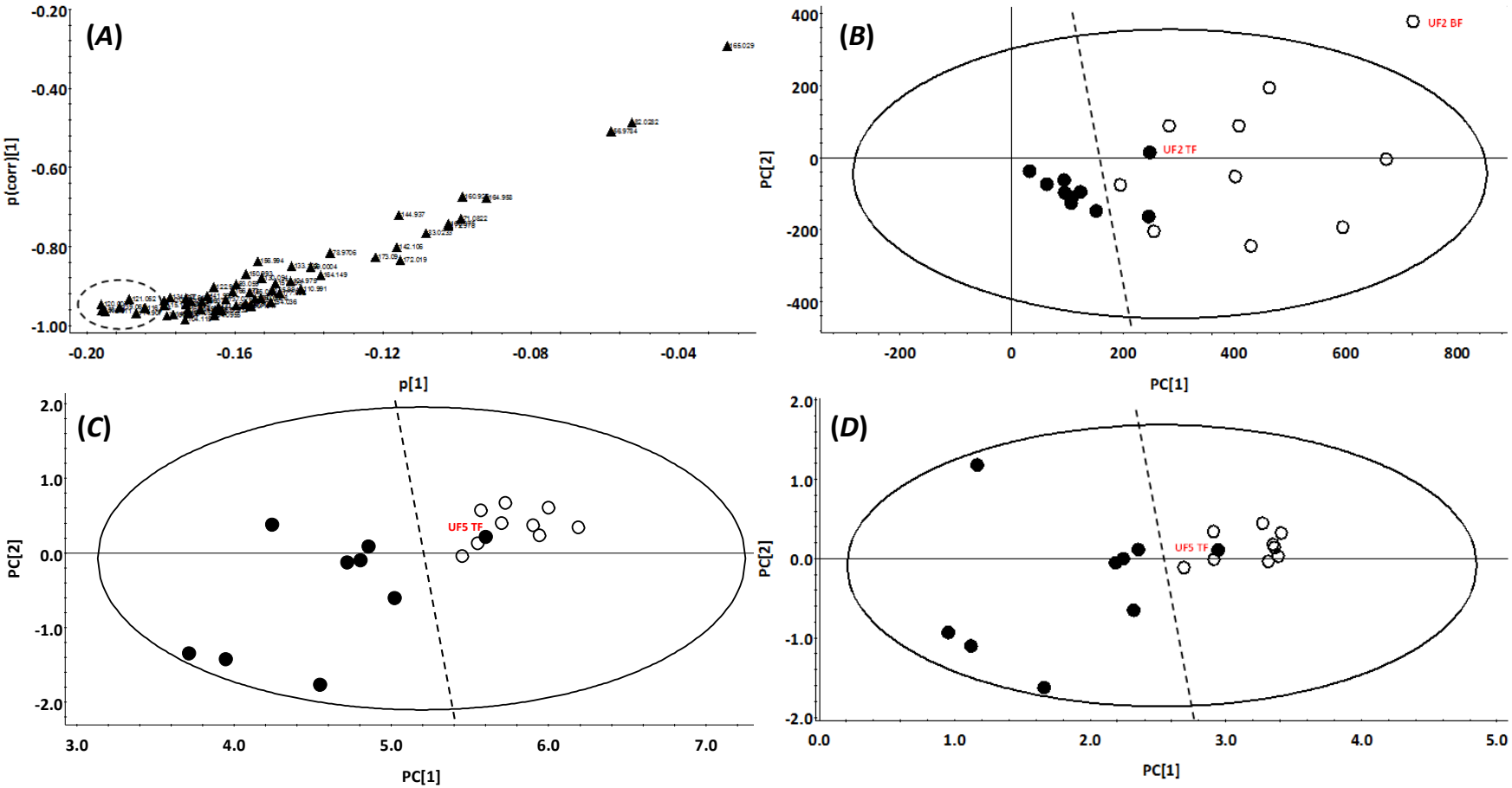


Figure 5.13. (A) PLS-DA S-plot obtained from 20 observations analysed by ESI-MS in positive ion, R^2X = 86.3%, Q^2X = 47.7%. Unsupervised PCA of LSP skin sensitive ions identified from PLS-DA on (B) 20 observations and (C) 18 observations, in positive ion mode. 7 ions were used to model the scatter plot from 61 ions identified from PLS-DA. (D) Unsupervised PCA of LSP skin sensitive ions identified from PLS-DA model on 18 observations in positive ion mode. 2 ions (m/z 114.9070 and 116.0842) were used to model the scatter plot from 34 ions identified from PLS-DA. 90% sensitivity was obtained for the plantar (BF) sample site observations and 100% specificity was obtained for the dorsal (TF) sample site in negative ion mode. R^2X = 100%, Q^2X = 100%. Dots = dorsal (TF) observations, circles = plantar (BF) observation.

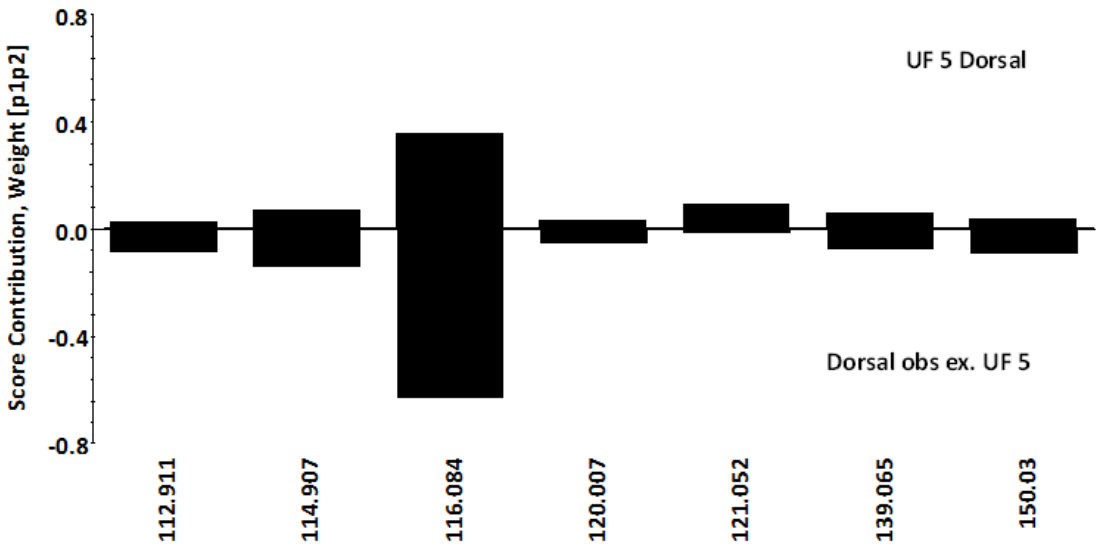


Figure 5.14. PCA ion contribution plot from dorsal (TF) observations from Figure 5.13 (C). Top bars show ion contribution for UF5 Dorsal (TF) sample whereas bottom bars show contribution from all other dorsal (TF) observations

For all ions used for the model above the UF5 dorsal (TF) observation has ion weight contributions closer to the plantar (BF) samples compared to other dorsal (TF) observations hence UF5 dorsal (TF) is observed away from the dorsal (TF) cluster. By removing one ion at a time, a model showing the separation between the dorsal (TF) and plantar (BF) sample site groups was observed with only two ions used in the data set. Ions (variables) with m/z 114.9070 and 116.0842 produced a model consisting of two significant components using eighteen observations with a cumulative variation (R^2X) of 100% of which could be cross validated (Q^2X) and 100% sensitivity and specificity (Figure 5.13 (D)).

Descriptive Statistics

Using the intensities obtained for each ion the differences between the dorsal (TF) and plantar (BF) samples were calculated for all sixty one observed ions from the average obtained from nine participants (excluding participant UF2). ANOVA was performed on the data set to discover whether any statistical differences in variance could be found. At 95%, ANOVA provided a calculated F value of 12.4 between the sample sites (Appendix Table 8.18). This value was much larger than the critical F value of 1.33 thus evidence of significant difference between the sample sites was present. Following this a paired t-test was carried out, on normalised data (log_{10} of original value) due to the data set not being from a normal distribution, to determine whether the two sample sites were significantly different (Table 5.12). H_0: no difference in intensity obtained from dorsal (TF) and plantar (BF) samples.

The largest difference between the dorsal (TF) and plantar (BF) sample sites was found for the ion m/z 116.0842 with a difference of 42095 counts. Statistically significant evidence of differences between the two sample sites for the ion m/z 116.0842 was found at 99% using the paired t-test. Other large differences were found with ions m/z 112.9112, 150.0297, 120.0073 and 139.0653 with intensities obtained at 30530, 30418, 26592 and 20963, respectively. There was also evidence of differences between the two sample sites at 99% using a paired t-test for all four of these ions. Ion m/z 150.0297 also showed significant difference between the two sites at 99.9%. The smallest difference of intensity between the plantar (BF) and dorsal (TF) sample sites was found for ions m/z 82.0282, 56.9784 and 165.0289. For these three ions, no statistically significant evidence of differences between the two sample sites was discovered. Plantar (BF) intensities obtained were higher than dorsal (TF) intensities in fifty nine of the sixty one ions whereas for ions m/z 99.0004 and 165.0289 the dorsal (TF) intensities were found to be slightly higher than the plantar (BF) intensities. However, for both of these ions no statistically significant evidence of differences was determined between the two sample sites using a paired t-test. From the sixty one ions, using a paired t-test, significant differences were found between the plantar (BF) and dorsal (TF) sample sites for thirty nine ions at 95%, twenty two ions at 99% and ten ions at 99.9% (Table 5.12).

Significance testing was also carried out to find whether gender differences existed (5 males, 4 females) between the two sample sites. Between male and female plantar (BF) samples differences were found in two of the sixty one ions at 95%, ions with m/z 225.0034 (male = 7619, female = 1556) and 99.0004 (male = 4091, female = 726), with male higher than female intensities for both (Appendix Table 8.19).

Five of the sixty one ions were found have significant differences between gender for dorsal (TF) samples. Ions m/z 112.9112 (male = 24119, female = 2224), 99.0004 (male = 5594, female = 1), 148.9789 (male = 1263, female = 105), 98.9343 (male = 901, female = 207) and 90.1047 (male = 537, female = 87) were determined to have significant gender differences in the dorsal (TF) site at 95% and ions m/z 148.9789 was also discovered to have significant gender differences at 99% for the dorsal (TF) site (Appendix Table 8.20).

Accurate Mass Determination

Four plantar (BF) samples (UF1, UF4, UF5, and UF8) were analysed using nESI-MS in both negative and positive ion modes to identify potential molecular formulae for the ions. In negative ion mode thirty of the thirty five observed ions yielded possible matches, although

structural formulae were not determined (Table 5.11). In positive ion mode forty three of the sixty one ions were found to have potential matches (Table 5.12). The suggested empirical formulae obtained from accurate mass determination proposed that many of the obtained ions could contain nitrogen and sulphur. Ions containing nitrogen and oxygen could be nitrate (NO_3^-) or nitrite (NO_2^-) based compounds. These are known to be in bodily fluids and studies carried out proved that the amount of nitrate excreted in urine is higher than those in foods indicating that it is a product of biosynthesis.[92]

Table 5.12. Mean (μ), standard deviation (σ) and the difference for 61 ion intensities, of dorsal (TF) and plantar (BF) samples, obtained by ESI-MS n positive ion mode. Results of paired t-test (P_{calc}) and whether H_0 was accepted (✓) or rejected (✖) are shown. Participant 2 excluded from data set. Also shown are accurate ion mass, empirical formula, relative double bond equivalents (RDB), and mass error (δppm) obtained by nESI-MS in positive ion mode from four participant samples. Ions ordered by intensity difference between plantar (BF) and dorsal (TF) samples

Ion (m/z)	TF (Total)		BF (Total)		μ (BF) - μ (TF)	P_{calc}	H_0 accepted ($P_{calc}>P_{crit}$)			Ion (m/z)	Empirical formula	RDB	δppm
	μ	σ (n = 9)	μ	σ (n = 9)			P_{crit} (0.05)	P_{crit} (0.01)	P_{crit} (0.001)	Accurate Mass			
116.0842	1949	3881	44044	35600	42095	0.004	✖	✖	✓	116.0706	$C_5 H_{10} O_2 N$	1.5	-0.389
112.9112	13880	20336	44409	30715	30530	0.003	✖	✖	✓	112.8956			
150.0297	6238	5015	36656	18175	30418	0.000	✖	✖	✖	150.0134	$C_2 H_4 O_5 N_3$	2.5	-7.444
120.0073	14004	9106	40596	18378	26592	0.001	✖	✖	✓	120.0031	$C H_2 O_4 N_3$	2.5	-7.184
139.0653	7257	6428	28220	19001	20963	0.003	✖	✖	✓	139.0499	$C_6 H_7 O_2 N_2$	4.5	-2.114
114.907	6530	10601	21426	15438	14896	0.019	✖	✓	✓	114.0913	$C_6 H_{12} O N$	1.5	-0.794
110.0838	3482	4079	16056	14855	12574	0.053	✓	✓	✓	110.0713	$C_5 H_8 N_3$	3.5	0.419
130.0939	1003	1871	11922	16657	10920	0.038	✖	✓	✓	130.0861	$C_6 H_{12} O_2 N$	1.5	-1.27
115.1008	1383	1529	12255	7634	10872	0.010	✖	✖	✓	115.0866	$C_5 H_{11} O N_2$	1.5	-0.257
70.0783	889	1446	11067	10875	10177	0.013	✖	✓	✓	70.0655	$C_4 H_8 N$	1.5	5.626
155.0909	220	516	9739	17115	9519	0.000	✖	✖	✖	155.0787	$C_3 H_7 N_8$	4.5	-0.508
140.0849	803	511	9834	14202	9030	0.006	✖	✖	✓	140.0679	$C_3 H_6 N_7$	4.5	-0.141
122.9668	5101	6437	12006	8633	6905	0.057	✓	✓	✓				
121.052	15600	10183	21918	17586	6318	0.556	✓	✓	✓	121.0395	$C_6 H_5 O N_2$	5.5	-0.821
137.076	302	582	6543	4168	6241	0.000	✖	✖	✖	137.0683	$C_2 H_9 O_3 N_4$	0.5	9.873
138.0667	1271	1527	6702	4910	5432	0.020	✖	✓	✓	138.0523	$C_3 H_4 N_7$	5.5	-0.07
94.9435	4492	5104	9671	6153	5179	0.078	✓	✓	✓				
134.0272	4873	4308	10038	5988	5165	0.081	✓	✓	✓	134.0186	$C_2 H_4 O_4 N_3$	2.5	-7.701
165.9969	616	954	5776	4460	5160	0.001	✖	✖	✖	165.9874	$C_5 O_4 N_3$	7.5	-5.916
150.9935	5310	7385	10326	7414	5016	0.101	✓	✓	✓				
135.9943	449	679	4895	3148	4446	0.004	✖	✖	✓				
144.937	1616	2214	5268	5705	3652	0.255	✓	✓	✓				

114.0034	1313	1817	4924	4415	3611	0.009	×	×	✓	114.0661	$C_4 H_8 O N_3$	2.5	-1.126
141.9948	1557	1104	4995	2871	3438	0.048	×	✓	✓				
96.9377	1946	1716	5328	2671	3382	0.055	✓	✓	✓				
104.1145	747	1037	4053	1787	3305	0.005	×	×	✓	104.1071	$C_5 H_{14} O N$	-0.5	0.666
164.0496	626	552	3799	2051	3173	0.000	×	×	×	164.029	$C_3 H_6 O_5 N_3$	2.5	-7.174
156.9937	5698	3948	8785	5843	3087	0.182	✓	✓	✓	156.9845	$C_8 H N_2 S$	9.5	-6.531
160.0446	936	602	3968	1790	3032	0.000	×	×	×	160.0342	$C_4 H_6 O_4 N_3$	3.5	-7.075
84.0955	281	394	3185	2352	2904	0.004	×	×	✓	84.0811	$C_5 H_{10} N$	1.5	3.498
133.1022	1365	1828	4185	4189	2820	0.130	✓	✓	✓	133.0969	$C_5 H_{13} O_2 N_2$	0.5	-1.76
60.0578	413	493	3053	4303	2640	0.025	×	✓	✓	60.0562	$C H_6 N_3$	0.5	8.762
166.9725	2993	6018	5465	3998	2472	0.053	✓	✓	✓				
162.0714	950	648	3418	1445	2469	0.045	×	✓	✓	162.0498	$C_4 H_8 O_4 N_3$	2.5	-7.172
177.0821	163	342	2357	1123	2194	0.000	×	×	×	177.0607	$C_4 H_9 O_4 N_4$	2.5	-6.672
139.9322	972	1371	3109	2057	2137	0.021	×	✓	✓				
132.9958	2290	1271	4421	3175	2131	0.087	✓	✓	✓	132.9875	$C_2 H O_5 N_2$	3.5	-7.126
148.9789	748	1067	2819	2417	2070	0.013	×	✓	✓	149.0231	$C_8 H_5 O_3$	6.5	-1.748
154.0362	109	232	2116	1357	2007	0.000	×	×	×	154.0261	$C_5 H_4 O_3 N_3$	5.5	8.846
157.0029	2031	3686	3999	5330	1968	0.036	×	✓	✓	157.0832	$C_4 H_9 O N_6$	3.5	-0.353
90.1047	337	457	2196	1119	1859	0.006	×	×	✓	90.0916	$C_4 H_{12} O N$	-0.5	2.991
173.0899	51	77	1394	1131	1344	0.000	×	×	×	173.0781	$C_4 H_9 O_2 N_6$	3.5	-0.405
164.9579	666	1995	1968	3106	1302	0.035	×	✓	✓	164.9202			
176.092	353	199	1599	862	1246	0.001	×	×	×				
93.059	1677	1077	2891	2554	1214	0.737	✓	✓	✓	93.0449	$C_5 H_5 N_2$	4.5	2.098
98.9343	592	476	1724	788	1132	0.043	×	✓	✓	98.9755	$H_3 O_4 S$	-0.5	8.327
78.9706	1512	1496	2474	2185	962	0.670	✓	✓	✓	79.0182	$C_5 H_3 O$	4.5	4.54
110.9912	276	490	1206	1212	930	0.013	×	✓	✓				
71.0822	81	175	980	2354	899	0.087	✓	✓	✓	71.0859	C_5 H11	0.5	5.53
124.975	979	1047	1795	1364	815	0.053	✓	✓	✓				

142.1064	70	178	823	895	754	0.018	×	✓	✓	142.0471	$C_2 H_4 O N_7$	4.5	-0.594
99.0004	3108	5041	2464	3215	644	0.102	✓	✓	✓	98.9843			
164.1488	823	1975	1436	2588	612	0.046	×	✓	✓	164.0915	$C_6 H_{14} O_4 N$	0.5	-1.49
83.0233	338	530	842	838	504	0.056	✓	✓	✓	83.0256			
172.0192	62	105	522	337	460	0.004	×	×	✓	171.9953	$C_8 H_2 N_3 S$	9.5	-6.421
160.9231	822	1355	1193	1328	370	0.554	✓	✓	✓	161.0318	$C_4 H N_8$	8.5	-0.55
172.9783	315	767	682	638	367	0.030	×	✓	✓	172.9959	$H_5 O_5 N_4 S$	0.5	-9.286
168.9755	295	882	501	589	206	0.041	×	✓	✓	168.9399	$C_5 H O_3 N_2 S$	6.5	-2.125
165.0289	174	451	1	0	173	0.175	✓	✓	✓	165.0067	$C_2 H_5 O_3 N_4 S$	2.5	-6.104
56.9784	285	749	370	1000	85	0.928	✓	✓	✓				
82.0282	123	323	130	276	7	0.917	✓	✓	✓	83.0219			
						H_0 accepted	22	39	51				
						H_0 rejected	39	22	10				
						Total	61	61	61				

5.9 Conclusion

The method development on the LSP presented in this chapter proves that this skin sampling device is able to sample human skin. The experiment in section 5.4 showed that the LSP concept achieved significant extraction of a selected sample when compared to baseline levels. Using a sampling solvent of EDTA the LSP was able to detect a drop of Mn that was spiked onto the surface of a nitrile glove. Although the first sample taken using the LSP achieved the greatest recovery, multiple washes were needed to extract the remaining sample from the sample surface. 4 samples containing Mn were collected with a 41.8% recovery for the first sample, 13.5% recovery for the first wash sample, 6.7% recovery for the second wash sample and 3.2% for the final wash sample.

For the next experiment (section 5.5) three sampling solvents were chosen to recover known amounts of a selected sample that was placed on a target sample surface, using the LSP. The sampling solvents used were EDTA, 2% HNO_3 and DI H_2O and the highest recovery was obtained for samples collected using 2% HNO_3 (55.7% recovery for the first sample) as the sampling solvent, followed by EDTA (39.9% recovery for the first sample) and then DI H_2O (18.6% recovery for the first sample). Out of the three sampling solvents, EDTA was chosen as the sampling solvent for future experiments despite 2% HNO_3 exhibiting higher recoveries. This was primarily due to the unsuitability of HNO_3 with the human skin which could cause potential health risks such as burning and irritation. EDTA was considered a more appropriate solvent as it was more compatible with human skin and was a good chelating agent to extract metals from the skin surface.

The first LSP study involving human participants is presented in section 5.6. Samples were collected using EDTA, as the sampling solvent, and analysed using ICP-MS. 8 elements were recovered from the volar (AE) and plantar (BF) skin sample sites using the LSP. No statistically significant evidence differences in elemental recoveries between the two skin sample sites were discovered. It was believed that high levels of Si found in the samples was due to elemental leeching from the PEEK tubes that transported the sample to and from the target skin surface. Consequently, further investigations into the method of cleaning the LSP's prior to human skin sampling, were successfully sought.

The LSP was used to detect, distinguish and recover elements from four parts of human skin. Four skin sampling sites on ten participants (five male and five female) were investigating using the LSP in section 5.7. 6 elements were detected over the four sites with the element K having the highest amount in each site. K was found to be highest in the dorsal (TF) and

plantar (BF) sites when compared to the axilla (AP) and volar (AE). S, Mg, Fe and Al were also detected as well as a small amount of Cu. There was also significant differences (95%) discovered for K between axilla (AP) and dorsal (TF) sample sites, for Mg between plantar (BF) and volar (AE) sites, for S from the volar (AE) sample site between male and females, and Mg in the axilla (AP) between the right and left sample site. Gender differences in S were also observed as well as Mg differences between the sides sampled in the axilla (AP).

Semi-VOCs and VOCs were investigated on skin sample sites at the top and bottom of human feet, namely the dorsal (TF) and plantar (BF), respectively. A total of ten participants (five male and five female) were sampled in the study discussed in section 5.8 where 96 ions were detected using ESI-MS. 60 of the 96 ions displayed significant differences between the two sample sites. Gender differences were also proven for a number of ions determined. High resolution analysis by nESI-MS using the Thermo Exacutive Orbitrap proposed ions could contain nitrites, nitrates and sulphur. Although nESI-MS provided accurate mass determination, these ions could not be verified due to lack of resources and time. A number of possible structures were available for each ion using the instrument libraries. The requirement of hundreds of reference samples to prove ion structures was not feasible either financially or in programme time.

Future optimisation of the LSP is required. Different pH levels of EDTA could be studied by the addition of a masking agent and then investigating the amount of sample recovered from known amounts spiked onto a surface. Anions such as phosphates will interfere with the selectivity of EDTA so these must be removed prior to analysis. Metal ions as well as EDTA are pH dependant, and for divalent ions, solutions must be kept basic for the reaction to go to completion. Many metal ions (e.g. Ca or Mg) only react completed when EDTA is in pure anionic form ($EDTA^{4-}$). Since it is a quadruple acid this can only be realised at pH value above 10 for Ca^{2+} ions, for Mg ions even higher pH > 12. In additional, a vibrating motor could be added to the tip of the pen in order to agitate the skin surface with the anticipation of better efficiency. Further development of the LSP should continue to enable elemental, semi-VOC and VOC mapping of the human body. Different sample heads could be trialled in an attempt to optimise sample recovery from parts of the body that are more difficult to sample such as the head. Following this, development into real-time analysis of human skin should be the aim for the use of the LSP. The LSP can then be used on individuals that have a stress condition to determine the biomarkers of stress on human skin from different sites around the body.

6 Conclusions

The objective of this research was to identify differences in the chemical profiles of biological fluids in the human body obtained from individuals under a variety of stress states e.g. physically or mentally. These profiles were collected by analysing biological fluids such as blood, saliva, sweat and urine that were attained by both invasive and non-invasive sampling techniques. Samples were analysed using a variety of techniques including inductively coupled plasma mass spectrometry (ICP-MS), thermal desorption gas chromatography mass spectrometry (TD-GC-MS) and high performance liquid chromatography-ion mobility mass spectrometry (UPLC-IM-MS). Further to this, a skin sampling device was engineered as a potential new collection method to acquire skin samples from stressed individuals.

In the initial experiment forehead skin samples were obtained, using 3M™ Tegaderm™ +pads, from individuals participating in an experiment containing an intervention designed to induce psychological stress. Individuals were required for this double cross over randomised design with two experimental interventions. One intervention required participants to listen to classical music, which was chosen to be neutral, while the other required participants to undertake a PASAT that induced cardiovascular responses consistent with acute stress. Forehead skin samples were taken from 22 participants (10 male and 10 female) using patches which were analysed for changes in elemental concentrations by ICP-MS. A new formula was devised in order to score stress based upon cardiovascular output. During the PASAT intervention an increase in cardiovascular function, as a consequence of stress resulting in increased intensity in heart rate and blood pressure, triggered the heating of the body inducing sweat onto the skin. This higher than normal skin temperature was exhibited in GSR readings where an increase level was observed during the PASAT intervention.

The analysis of the elemental content in these skin patch samples was performed using ICP-MS following sample collection. Although 11 elements were detected it was discovered that the patches used contained high levels of elemental impurities. Consequently, the elemental content of patches was to be reduced through solvent washes prior to collecting skin samples in future studies. Consequently, MVA did not exhibit any separation. A repeat of this study using up to fifty participants and patches reduced in elemental impurities should be conducted in order to identify elemental markers of psychological stress. Any future repeats of this study should comprise of new participants in order to remove any bias of memory effects of the PASAT from participants who have already taken the test.

Elemental impurities were removed from patches using a series of solvent washes. Patches were washed in 15% HNO_3 for 6 hours to remove impurities with basic properties, such as alkali's that react with water forming X-OH e.g. LiOH, NaOH, KOH). A wash with 10% TMAH was conducted to remove any acid-soluble impurities i.e. oxides formed with elements with low electron affinity e.g. SiO_2, SO_2, SO_3 etc.; DI H_2O. Next, 0.1M EDTA was used to remove any metal impurities by the chelating effect followed by a DI H_2O wash in order to neutralise the patches so cleaned patches would not pose any risk to participant skin. In between each wash, patches were immersed in DI H_2O and dried under IR.

The second study saw ten male participants take part in an experiment with an intervention designed to replicate physical stress. A variety of biological samples were collected prior to and following a thirty minute exercise using a cycle ergometer set at a resistance of 2 $W.Kg^{-1}$ of participant body mass. The detection of 10 elements in forehead skin (Na, Mg, Al, Si, P, S, K, Ca, Cu, Zn), 8 elements in drool saliva (Na, Mg, Si, P, S, K, Zn, I), 8 elements in urine (Na, Mg, Si, P, S, K, Ca, Zn, Mo) and 7 elements in blood plasma (Mg, P, K, Cu, Zn, Li, Fe) were detected. Elemental changes were discovered in forehead skin, urine and drool saliva following stress. No changes were observed in plasma. Data was initially analysed using MVA producing statistical models which indicated possible elemental markers of stress. Significance testing was then used to confirm whether these biomarkers could be statistically scrutinized using commonly used data analysis methods. A physical stress score, based using the previous formula invented for psychological stress, was also designed which exhibited participant stress levels. Incidentally, this correlated with how participants rated their own stress levels. Using 4 globally detected elements (Mg, P, K, Zn) from the four biological samples, MVA was used to identify the major contributors of physical stress and found significant contributors from forehead skin and urine.

Further to this, data obtained from saliva analysed using TD-GC-MS, UPLC-IM-MS and ICP-MS was grouped into one data set and analysed by MVA resulting in a super score plot displaying the capability of discovering biomarkers of stress from a variety of techniques and presenting them in one statistical model. Although stress states of participants in saliva could be established using these techniques there was difficulty in identifying the discovered markers due to multiple possibilities of ion identities from instrument libraries for discovered masses. Purchasing standards for each ion possibility would not be financially feasible and would prove very time consuming. Consequently, further studies should be conducted in order to confirm similar biomarkers of stress after which standards should then be purchased to identify these biomarkers.

Further work includes the analysis of all samples collected from the study. Upon data analysis of these samples, the questions posed should include which areas of the human body are substantially affected by stress and can pathways of these areas be deduced, showing the first effected areas. Once biomarkers are identified there could be a possibility to develop counter acting drugs of stress which could be trialled on human volunteers who are physically stressed.

An opportunity was taken to collect saliva in an experiment designed to replicate a trapped human in a collapsed building inducing emotion stress. The intervention included the participant being placed in an entrapped space for twenty four hours with no food or water. Drool saliva was collected prior to and after the intervention and samples were analysed for elemental changes by ICP-MS. 9 possible elemental markers were discovered with the largest changes detected for Na and I while large changes were also found for Mn, Fe and P. Changes for Sr, As, Mg and Si were also observed. It is believed that the presence of Sr was due to naturally occurring amounts of the element in human teeth which tend to leech Sr to aid the prevention of mouth diseases. Little information can be deduced from this study as only one participant was sampled. To confirm whether the elements are markers of emotional stress, further studies must be conducted using at least thirty participants.

Future studies should compare the discovered biomarkers from these different stress states using participants who undergo each stress intervention. If any biomarker is detected in all three stress studies then it should be closely inspected as it could be a significant biomarker of stress. Further development to suppress this biomarker should then be investigated in order to try and eliminate stress.

Finally, the design and construction of a skin sampling device is shown. The Liquid Skin Pen (LSP) is capable of acting as a rapid, non-invasive way to collect skin elements and small molecules. Analysis of samples collected using the device demonstrated that different body parts may be consistently distinguished, and that a wide range of compounds can be collected and measured.

Initial tests showed successful recovery of Mn spiked onto a sample surface, using the LSP. Following this the comparison between three sampling solvents were tested. The results indicated that the highest recoveries were detected for 2% HNO_3 (57.7%) followed by 0.1M EDTA (39.9%) and then DI H_2O (18.6%). However, due to the requirement to use the LSP on human volunteers in further studies, it was much safer to use 0.1M EDTA as the sampling

solvent rather than the corrosive HNO_3. Therefore 0.1M EDTA was selected as the sampling solvent for subsequent studies using the LSP due to achieving higher recoveries then DI H_2O.

The first human experiments using the LSP involved sampling the volar and plantar skin sites. 8 elements were recovered but no statistically significant evidence of differences in elemental recoveries between the two skin sites was determined. High levels of Si were also detected and it is believed this presence was due to the leaching from the PEEK transport tubes used to carry the samples to and from vials via the target surface. Consequently, a new cleaning procedure was produced to eradicate memory effects of previous samples and increase efficiency of sample collection using LSP. This process included cleaning the LSP using the sampling solvent to be employed by simply flushing it through the transport tubes of the LSP's prior to sample collection. The new cleaning procedure was designed, tested and analysed for targeted small molecule ions after which it was utilised for subsequent studies.

The next human experiment using the LSP involved investigating the differences between the axilla (AP), dorsal (TF), plantar (BF) and volar (AE). A total of 6 elements were detected across these four sites with K observed in each site and differences in elemental concentrations discovered between all four sites. The highest elemental concentrations were found for K in the dorsal (TF) and plantar (BF). Amounts of the elements S, Mg, Fe, Al and Cu were also detected. Gender differences in S were discovered as well as Mg differences between the body sides sampled in the axilla.

MVA was able to identify potential small molecules significant to the dorsal (TF) or plantar (BF) of the foot. 96 ions were detected from both positive and negative ion mode using electrospray ionisation mass spectrometry (ESI-MS). 60 of these 96 ions displayed significant differences between the two sample sites whilst gender differences were also determined for a number of ions. Accurate mass determination by nano-electrospray ionisation mass spectrometry (nESI-MS) of samples suggested that many compounds discovered in the dorsal (TF) and plantar (BF) of human feet could contain nitrites, nitrates and sulphur. However, these suggested compounds from the library could not be identified due to the lack of standard availability and time constraints.

This proof-of-concept study demonstrates that the LSP is able to effectively collect elements and small molecules from a variety of sites on the human skin where samples obtained can be analysed by ESI-MS and ICP-MS. The LSP should be further developed in order to optimise the efficiency of sample recovery using a variety of solvents for small molecule analysis or by

experimenting with the solvent buffer for elemental analysis. Different bases on the device should be tested in order to sample all parts of the body including the head. A vibrating motor could be added in the base in order to agitate molecules on the skin possibly providing greater efficiency. Further work includes full body mapping using the LSP followed by online analysis showing real time data from human skin. Furthermore, the LSP could be employed in stress studies to collect skin samples where data from samples obtained using the device could be compared against skin data from other skin sampling techniques such as using a PDMS membrane, acid wipes or rinsing.

7 References

1 C. L. Cooper and P. J. Dewe, *Stress: A Brief History*, Blackwell Publishing Ltd, Oxford, Uk, 2004.
2 L. Goldberger and S. Breznits, *Handbook of Stress: Theoretical and Clinical Aspects*, Free Press, 1982.
3 H. Selye, *The Stress of Life*, New York: McGraw-Hill, 1956.
4 G. Brengelmann and B. AC, *Temperature regulation. In: Rush TC, Patton HD (eds) Physiology and biophysics*, W B Saunders company, Philadelphia and London, 1965.
5 T. Ogawa and J. Sugenoya, *Japanese J. Physiol.*, 1993, 43, 275–289.
6 J. Sugenoya, T. Ogawa, M. Asayama, and T. Miyagawa, *Japanese J. Physiol.*, 1982, 32, 717–726.
7 Y. Ando, S. Araki, O. Shimoda, and T. Kano, *Muscle Nerve*, 1992, 15, 507–512.
8 S. Homma, K. Matsunami, X. Y. Han, and K. Deguchi, *Neurosci. Lett.*, 2001, 305, 1–4.
9 M. Kobayashi, N. Tomioka, Y. Ushiyama, and T. Ohhashi, *Auton. Neurosci.*, 2003, 104, 58–65.
10 K. Matsuda, H. Kobayashi, and S. Watanuki, *Appl. Hum. Sci.*, 1996, 15, 75–80.
11 O. Shimoda and Y. Ikuta, *Clin. Auton. Res.*, 2005, 15, 408–10.
12 T. Kamei, T. Tsuda, S. Kitagawa, K. Naitoh, and T. Ohhashi, *Anal. Chim. Acta*, 1998, 365, 319–326.
13 H. Storm, *Early Hum. Dev.*, 2001, 62, 149–58.
14 S. Abrams, *Am. J. Psychiatry*, 1973, 130, 94–98.
15 C. G. Jung, *J. Abnorm. Psychol.*, 1907, 2, 247–255.
16 M. V. Villarejo, B. G. Zapirain, and A. M. Zorrilla, *Sensors (Basel).*, 2012, 12, 6075–101.
17 N. Nourbakhsh, Y. Wang, F. Chen, and R. A. Calvo, *Using Galvanic Skin Response for Cognitive Load Measurement in Arithmetic and Reading Tasks The University of Sydney*, New York, NY, USA, 2012.
18 M. W. J. Dodds, D. A. Johnson, and C.-K. Yeh, *J. Dent.*, 2005, 33, 223–33.
19 P. R. N. Sutton, *Stress and dental caries. In stable PH (ed), Advances in Oral Biology 2*, Academic Press, New York, 1966.
20 D. R. Morse, G. R. Schacterle, M. L. Furst, J. V. Esposito, and M. Zaydenburg, *Ann. Denistry*, 1983, 42, 47–54.
21 S. Cohen and G. M. Williamson, *Psychol. Bull.*, 1991, 109, 5–24.
22 I. Eli, *Oral psychophysiology - Stress, pain and behaviour in dental care*, CRC Press, Boca Raton, FL, 1992.
23 W. S. Marcenes, R. Croucher, A. Sheiham, and M. Marmot, in *Psychological Health 8*, 1993, pp. 123–134.
24 P. C. Fox, P. F. Van der Ven, B. . Sonies, J. M. Weiffenbach, and B. J. Baum, *J. Am. Dent. Assoc.*, 1985, 10, 519–525.
25 C. Dawes, *J. Dent. Res.*, 1987, 66, 648–653.
26 J. A. Bosch, H. S. Brand, T. J. M. Ligtenberg, B. Bermond, J. Hoogdtraten, and N. Amerongen, *Psychosom. Med.*, 1996, 58, 374–382.
27 K. C. Madison, *J. Invest. Dermatol.*, 2003, 121, 231–241.
28 E. Proksch, J. M. Brandner, and J.-M. Jensen, *Exp. Dermatol.*, 2008, 17, 1063–1072.
29 W. L. Kenney and T. a Munce, *J. Appl. Physiol.*, 2003, 95, 2598–2603.
30 C.-T. Huang, M.-L. Chen, L.-L. Huang, and I.-F. Mao, *Chin. J. Physiol.*, 2002, 45, 109–15.
31 O. Engelsen, M. Brustad, L. Aksnes, and E. Lund, *Photochem. Photobiol.*, 2005, 81, 1287–90.
32 M. I. Haq, E. Smith, D. N. John, M. Kalavala, C. Edwards, a Anstey, a Morrissey, and J. C. Birchall, *Biomed. Microdevices*, 2009, 11, 35–47.
33 J. Varani, *Histol Histopathol*, 1998, 13, 775–783.

34 M. Katona, J. Dénes, R. Skoumal, M. Tóth, and Z. Takáts, *Analyst*, 2011, 136, 835–40.
35 S. Riazanskaia, G. Blackburn, M. Harker, D. Taylor, and C. L. P. Thomas, *Analyst*, 2008, 133, 1020–7.
36 C. Lidén, L. Skare, B. Lind, G. Nise, and M. Vahter, *Contact Dermatitis*, 2006, 54, 233–8.
37 A. L. Stinchcomb, F. Pirot, G. D. Touraille, A. L. Bunge, and R. H. Guy, *Pharm. Res.*, 1999, 16, 1288–1293.
38 P. Dube, C. Krause, and L. Windmüller, *Analyst*, 1989, 114, 1249–53.
39 J. P. Snell, S. Sandberg, and W. Frech, *J. Anal. At. Spectrom.*, 1997, 12, 491–494.
40 C. S. Muñiz, J. M. Marchante-Gayón, J. I. G. Alonso, and A. Sanz-Medel, *J. Anal. At. Spectrom.*, 1998, 13, 283–287.
41 E. H. Evans and J. J. Giglio, *J. Anal. At. Spectrom.*, 1993, 8, 1.
42 H. Vanhoe, J. Goossens, L. Moens, and R. Dams, *J. Anal. At. Spectrom.*, 1994, 9, 177.
43 C. S. Hsiung, J. D. Andrade, R. Costa, and K. O. Ash, *Clin. Chem.*, 1997, 43, 2303–11.
44 E. Barany, I. A. Bergdahl, A. Schütz, S. Skerfving, and A. Oskarsson, *J. Anal. At. Spectrom.*, 1997, 12, 1005–1009.
45 D. E. Nixon, M. F. Burritt, and T. P. Moyer, *J. Anal. Toxicol.*, 2007, 31, 281–7.
46 J. J. Thompson and R. S. Houk, *J. Appl. Spectrosc.*, 1987, 41, 801–806.
47 I. Feldmann, W. Tittes, N. Jakubowski, D. Stuewer, and U. Giessmann, *J. Anal. At. Spectrom.*, 1994, 9, 1007.
48 W. Tittes, N. Jakubowski, D. Stüwer, G. Tölg, and J. A. C. Broekaert, *J. Anal. At. Spectrom.*, 1994, 9, 1015.
49 L. Moens, P. Verrept, R. Dams, U. Greb, G. Jung, and B. Laser, *J. Anal. At. Spectrom.*, 1994, 9, 1075.
50 L. Moens, F. Vanhaecke, J. Riondato, and R. Dams, *J. Anal. At. Spectrom.*, 1995, 10, 569.
51 R. M. Barnes, *Anal. Bioanal. Chem.*, 1996, 355, 433–41.
52 M. Patriarca, *Microchem. J.*, 1996, 54, 262–271.
53 J. Begerow and L. Dunemann, *J. Anal. At. Spectrom.*, 1996, 11, 303.
54 J. Begerow, *J. Anal. At. Spectrom.*, 1997, 12, 1095–1098.
55 M. Caddia and B. S. Iversen, *J. Anal. At. Spectrom.*, 1998, 13, 309–313.
56 S. Stürup, M. Hansen, and C. Mølgaard, *J. Anal. At. Spectrom.*, 1997, 12, 919–923.
57 J. Riondato, F. Vanhaecke, L. Moens, and R. Dams, *J. Anal. At. Spectrom.*, 1997, 12, 933–937.
58 R. Dams, J. Goossens, and L. Moens, *Mikrochim. Acta*, 1995, 119, 277–286.
59 M. Gerotto, *Microchem. J.*, 1995, 51, 73–87.
60 P. F. Infante and S. Newman, *Lancet*, 2004, 363, 415–416.
61 *Second National Report on Human Exposure to Environmental Chemicals: National Center for Health Division of Laboratory Sciences*, Atlanta, GA, 2003.
62 G. D. Nuyts, P. C. D'Haese, M. M. Elseviers, M. E. de Broe, E. Van Vlem, J. Thys, and D. De Leersnijder, *Lancet*, 1995, 346, 7–11.
63 G. D. Clayton and F. E. Clayton, *Patty's Industrial Hygiene and Toxicology*, John Wiley & Sons Ltd, New York, 4th edn., 1994.
64 C. Rey, H. J. Reinecke, and R. Besser, *Vet. Hum. Toxicol.*, 1984, 26, 121–122.
65 D. J. Douglas and J. B. French, *Spectrochim. Acta*, 1986, 41B, 197–204.
66 J. B. Fenn, M. Mann, C. K. A. I. Meng, S. F. Wong, and C. M. Whitehouse, *Science (80-.).*, 1989, 246, 64–71.
67 M. Dole, L. L. Mack, and R. L. Hines, *J. Chem. Phys.*, 1968, 49, 2240.
68 P. Kebarle and M. Peschke, *Anal. Chim. Acta*, 2000, 406, 11–35.
69 E. De Hoffmann and V. Stroobant, *Mass Spectrometry - Priniples and Applications.*, John Wiley & Sons Ltd, Third., 2007, vol. 29.

70 A. Gomez and K. Tang, *Phys. Fluids*, 1994, 6, 404.
71 T. C. Rohner, N. Lion, and H. H. Girault, *Phys. Chem. Chem. Phys.*, 2004, 3056–3068.
72 H. Chen, A. Venter, and R. G. Cooks, *Chem. Commun. (Camb).*, 2006, 2042–4.
73 A. Makarov, 1999, Mass Spectrometer. US Patent, 5886346.
74 A. Makarov, M. E. Hardman, J. C. Schwartz, and M. Senko, 2004, US Patent, 2004108450.
75 A. Makarov, *Anal. Chem.*, 2000, 72, 1156–62.
76 J. N. Miller and J. C. Miller, *Statistics and Chemometrics for Analytical Chemistry*, Pearson Education Limited, Fifth., 2005.
77 A. M. Weljie, J. Newton, P. Mercier, E. Carlson, and C. M. Slupsky, *Anal. Chem.*, 2006, 78, 4430–42.
78 Z. Ramadan, D. Jacobs, M. Grigorov, and S. Kochhar, *Talanta*, 2006, 68, 1683–91.
79 D. M. Gronwall and H. D. Sampson, *The psychological effects of concussion*, Auckland University Press, Auckland and Wellington, 1974.
80 D. Carroll, A. C. Phillips, K. Hunt, and G. Der, *Biol. Psychol.*, 2007, 75, 68–74.
81 M. T. Feldner, E. W. Leen-Feldner, M. J. Zvolensky, and C. W. Lejuez, *J. Behav. Ther. Exp. Psychiatry*, 2006, 37, 171–87.
82 A. Steptoe, M. Hamer, and Y. Chida, *Brain. Behav. Immun.*, 2007, 21, 901–12.
83 I. Pavlidis, *Eng. Med. Biol. Soc.*, 2003, 2, 1084–1087.
84 R. Salvesen, *J. Neurol. Sci.*, 2001, 183, 39–42.
85 R. H. Rosenman, *Integr. Physiol. Behav. Sci.*, 1991, 26, 296–304.
86 J. E. Naschitz, I. Rosner, N. Shaviv, I. Khorshidi, S. Sundick, H. Isseroff, M. Fields, R. M. Priselac, D. Yeshurun, E. Sabo, and R. Itzhak, *J. Hum. Hypertens.*, 2003, 17, 111–8.
87 M. a Turner, S. Bandelow, L. Edwards, P. Patel, H. J. Martin, I. D. Wilson, and C. L. P. Thomas, *J. Breath Res.*, 2013, 7, 017102.
88 B. Gunnar, *Borg's Perceived Exertion and Pain Scales*, Human Kinetics, 1998.
89 S. Venturi and M. Venturi, *Nutr. Health*, 2009, 20, 119–134.
90 A. Surdacka, J. Stopa, and L. Torlinski, *Biol. Trace Elem. Res.*, 2007, 116, 147–53.
91 N. West, R. G. Newcombe, N. Hughes, S. Mason, B. Maggio, F. Sufi, and N. Claydon, *J. Dent.*, 2013, 41, 187–94.
92 H. H. Mitchell, H. A. Shonle, and H. S. Grindley, *J. Biol. Chem.*, 1916, 24, 461–490.

8 Appendices

Appendix I

Table 8.1. Calibration data for the eleven detected elements obtained from forehead skin patches by ICP-MS during the psychological stress study

Element	Gradient	Intercept	R^2	Standard Error	LOD	LOQ
Na	321912	34857846	0.9993	3374997	31	105
Zn	8704	52935	0.9995	40717	14.0	46.8
S	3859	144018	0.9995	19531	15.2	50.6
Sn	250	3335	0.9995	1259	15.1	50.4
Ca	2302	60970	0.9997	14337	18.7	62.3
K	14097	7898	0.9999	61999	13.2	44.0
Ba	113990	12712	0.9999	35063	0.9	3.1
Mg	20602	15273	1.0000	1285	0.2	0.6
Ti	3186	103	1.0000	379	0.4	1.2
Ni	12133	1434	1.0000	60	0.0	0.0
Cu	27593	5395	1.0000	1178	0.1	0.4

Very good linearity was obtained by ICP-MS for the eleven elements detected in sweat obtained from skin patches obtained from psychological stress. All coefficients of determinant values (R^2) are above 0.9990 suggesting the ICP-MS has been calibrated correctly and that the semi-quantitation of elements can be used to interpret the result. The linearity graphs for these eleven elements can be observed in Figure SI.4.1 in the supplementary information.

Table 8.2. ANOVA single factor for i) the stress session between male (MS) and female (FS) participants, ii) the neutral session between male (MN) and female (FN) participants and iii) between the stress (TS) and neutral (TN) session for all participants

Groups	*Count*	*Sum*	*Average*	*Variance*	*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Gender for stress session											
MS	10	462	46	2072	Between Groups	4419	1	4419	1.435	0.245	4.351
FS	12	896	75	3905	Within Groups	61601	20	3080			
					Total	66020	21				
Between Gender for neutral session											
MN	10	54	5	41	Between Groups	16	1	16	0.773	0.390	4.351
FN	12	44	4	5	Within Groups	427	20	21			
					Total	444	21				
Between stress and neutral session											
TS	22	1359	62	3144	Between Groups	36119	1	36119	**22.825**	0.000	4.073
TN	22	98	4	21	Within Groups	66464	42	1582			
					Total	102583	43				

The results for ANOVA show no evidence of significant differences in the variances between gender in the stress session and also in the neutral session as F_{calc} is smaller than F_{crit}. However, there is statistical evidence of a difference in variance between stress and neutral sessions for all participants as F_{crit} (22.8) is bigger the F_{crit} (4.07).

Appendix II

Table 8.3. Sample information summary for biological samples (with their intended instrument of analysis) and heart rate (HR) readings collected from physical stress study

Sample ID	Plasma		Saliva			Skin		Urine			Breath			HR readings*	Total
	ICP-MS	IMS	ICP-MS	IMS	VOC	ICP-MS	VOC	ICP-MS	IR-MS	IMS	GC-MS	GC-MS	DMS		
CZ1	3	3	3	3	3	4	4	3	3	3	3	3	10	18	66
CZ2	3	3	3	3	3	4	4	3	3	3	3	3	10	19	67
CZ3	3	3	2	3	3	4	4	3	3	3	3	3	10	17	64
CZ4	3	3	3	3	3	4	4	3	3	3	3	3	10	19	67
CZ5	3	3	3	3	3	4	4	3	3	3	3	3	10	20	68
CZ6	3	3	3	3	3	4	4	3	3	3	3	3	10	19	67
CZ7	3	3	2	3	3	4	4	3	3	3	3	3	10	19	66
CZ8	0	0	3	3	3	4	4	3	3	3	3	3	10	18	60
CZ9	1	1	3	3	3	4	4	3	3	3	3	3	10	21	65
CZ10	0	0	2	2	3	4	4	3	3	3	3	3	10	20	60
Total	22	22	27	29	30	40	40	30	30	30	30	30	100	190	***650***
	Plasma = 44		Saliva = 86			Skin = 80		Urine = 120			Breath = 130				

*HR includes experiment start & end, cycling start & end. Readings every 10 minutes phases A, C and D, every 5 minutes phase B.

Table 8.4. ANOVA (two way with replicates) for ten elemental skin patch concentrations (ng.mL^{-1}) obtained by ICP-MS from ten participants who participated in the physical stress study

	Na	Mg	Al	Si	P	S	K	Ca	Cu	Zn	Total
Phase A											
Count	10	10	10	10	10	10	10	10	10	10	100
Sum	4.63E+02	1.59E+01	1.85E+01	7.02E+01	2.59E+01	1.12E+02	8.39E+02	7.04E+02	1.70E+01	1.01E+03	3.28E+03
Average	4.63E+01	1.59E+00	1.85E+00	7.02E+00	2.59E+00	1.12E+01	8.39E+01	7.04E+01	1.70E+00	1.01E+02	3.28E+01
Variance	3.61E+03	1.50E+01	2.11E+01	1.52E+02	4.02E+01	2.59E+02	1.51E+04	3.06E+04	1.86E+01	7.43E+04	1.27E+04
Phase B											
Count	10	10	10	10	10	10	10	10	10	10	100
Sum	7.62E+04	6.97E+02	1.03E+02	6.09E+01	2.80E+02	3.03E+03	1.15E+05	2.75E+03	3.68E+01	3.72E+02	1.98E+05
Average	7.62E+03	6.97E+01	1.03E+01	6.09E+00	2.80E+01	3.03E+02	1.15E+04	2.75E+02	3.68E+00	3.72E+01	1.98E+03
Variance	1.71E+08	7.89E+02	8.28E+01	8.02E+01	3.98E+02	1.84E+04	4.03E+07	9.31E+03	2.81E+00	8.92E+03	3.45E+07
Phase C											
Count	10	10	10	10	10	10	10	10	10	10	100
Sum	3.37E+04	8.67E+01	2.81E+01	7.74E+01	7.58E+01	1.08E+03	1.11E+04	1.01E+03	1.04E+01	6.46E+01	4.73E+04
Average	3.37E+03	8.67E+00	2.81E+00	7.74E+00	7.58E+00	1.08E+02	1.11E+03	1.01E+02	1.04E+00	6.46E+00	4.73E+02
Variance	1.91E+07	3.15E+02	3.21E+01	1.58E+02	2.42E+02	7.89E+04	2.49E+06	4.96E+04	3.29E+00	1.25E+02	3.02E+06
Phase D											
Count	10	10	10	10	10	10	10	10	10	10	100
Sum	2.36E+03	8.40E+00	4.37E+01	8.67E+01	4.91E+00	9.11E+01	9.53E+02	2.60E+02	1.00E+00	6.05E+01	3.87E+03
Average	2.36E+02	8.40E-01	4.37E+00	8.67E+00	4.91E-01	9.11E+00	9.53E+01	2.60E+01	1.00E-01	6.05E+00	3.87E+01
Variance	2.47E+05	9.35E-01	3.27E+01	4.17E+02	1.53E+00	8.97E+01	2.48E+04	1.66E+03	2.14E-34	1.10E+02	3.00E+04
Total											
Count	40	40	40	40	40	40	40	40	40	40	
Sum	1.13E+05	8.08E+02	1.93E+02	2.95E+02	3.86E+02	4.32E+03	1.28E+05	4.72E+03	6.52E+01	1.51E+03	
Average	2.82E+03	2.02E+01	4.84E+00	7.38E+00	9.66E+00	1.08E+02	3.19E+03	1.18E+02	1.63E+00	3.77E+01	
Variance	5.37E+07	1.11E+03	5.00E+01	1.87E+02	2.79E+02	3.72E+04	3.35E+07	3.01E+04	7.48E+00	2.08E+04	
ANOVA											
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*					
Sample	2.56E+08	3	8.54E+07	**14.60**	5.39E-09	2.63					
Columns	5.67E+08	9	6.30E+07	**10.77**	7.65E-15	1.91					
Interaction	1.04E+09	27	3.87E+07	6.61	5.43E-19	1.52					
Within	2.10E+09	360	5.85E+06								
Total	3.97E+09	399									

Table 8.5. ANOVA (two way with replicates) for eight elemental drool saliva concentrations (ng.mL^{-1}) obtained by ICP-MS from ten participants who participated in the physical stress study

	Na	Mg	Si	P	S	K	Zn	I	Total
Phase A									
Count	7	7	7	7	7	7	7	7	56
Sum	2.99E+01	1.95E+01	1.30E+01	3.07E+01	2.65E+01	3.08E+01	8.31E+00	2.54E+01	1.84E+02
Average	4.26E+00	2.79E+00	1.86E+00	4.38E+00	3.78E+00	4.40E+00	1.19E+00	3.63E+00	3.29E+00
Variance	7.27E-02	8.29E-02	1.03E-01	2.82E-02	4.89E-02	7.40E-01	2.28E-01	1.99E-01	1.49E+00
Phase C									
Count	7	7	7	7	7	7	7	7	56
Sum	2.99E+01	1.96E+01	1.29E+01	3.03E+01	2.61E+01	3.03E+01	7.53E+00	2.40E+01	1.81E+02
Average	4.27E+00	2.80E+00	1.85E+00	4.33E+00	3.73E+00	4.33E+00	1.08E+00	3.42E+00	3.23E+00
Variance	8.35E-02	3.44E-02	1.23E-02	1.57E-02	2.42E-02	5.25E-01	3.79E-01	1.20E-01	1.47E+00
Phase D									
Count	7	7	7	7	7	7	7	7	56
Sum	3.03E+01	1.93E+01	1.24E+01	3.01E+01	2.56E+01	2.71E+01	8.75E+00	2.33E+01	1.77E+02
Average	4.32E+00	2.76E+00	1.77E+00	4.30E+00	3.66E+00	3.87E+00	1.25E+00	3.33E+00	3.16E+00
Variance	2.15E-02	2.97E-02	6.51E-02	8.85E-03	1.69E-02	8.12E-01	7.65E-02	8.95E-02	1.29E+00
Total									
Count	21	21	21	21	21	21	21	21	
Sum	9.00E+01	5.85E+01	3.83E+01	9.11E+01	7.82E+01	8.82E+01	2.46E+01	7.27E+01	
Average	4.29E+00	2.78E+00	1.83E+00	4.34E+00	3.72E+00	4.20E+00	1.17E+00	3.46E+00	
Variance	5.39E-02	4.45E-02	5.60E-02	1.70E-02	2.99E-02	6.81E-01	2.10E-01	1.39E-01	
ANOVA									
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*			
Sample	4.75E-01	2	2.37E-01	1.492	0.228	3.059			
Columns	2.10E+02	7	3.00E+01	188.592	0.000	2.074			
Interaction	1.26E+00	14	8.98E-02	0.565	0.889	1.761			
Within	2.29E+01	144	1.59E-01						
Total	2.35E+02	167							

Table 8.6. ANOVA (two way with replicates) for eight elemental urine concentrations (ng.mL^{-1}) obtained by ICP-MS from ten participants who participated in the physical stress study

	Mg	Si	P	S	K	Ca	Zn	Mo	Total
Phase A									
Count	10	10	10	10	10	10	10	10	80
Sum	1.90E+06	9.57E+04	1.61E+07	1.41E+07	4.03E+07	5.46E+05	3.18E+03	5.59E+02	7.31E+07
Average	1.90E+05	9.57E+03	1.61E+06	1.41E+06	4.03E+06	5.46E+04	3.18E+02	5.59E+01	9.14E+05
Variance	3.83E+10	4.95E+07	2.68E+12	1.08E+12	6.70E+12	1.94E+09	8.61E+04	2.01E+03	3.00E+12
Phase C									
Count	10	10	10	10	10	10	10	10	80
Sum	9.47E+05	6.65E+04	4.84E+06	8.45E+06	3.28E+07	3.14E+05	2.16E+03	3.29E+02	4.75E+07
Average	9.47E+04	6.65E+03	4.84E+05	8.45E+05	3.28E+06	3.14E+04	2.16E+02	3.29E+01	5.93E+05
Variance	9.57E+09	4.07E+07	4.06E+11	4.18E+11	3.97E+12	7.11E+08	4.28E+04	2.03E+03	1.68E+12
Phase D									
Count	10	10	10	10	10	10	10	10	80
Sum	3.22E+06	1.77E+05	1.15E+07	2.10E+07	5.66E+07	5.91E+05	6.06E+03	6.44E+02	9.31E+07
Average	3.22E+05	1.77E+04	1.15E+06	2.10E+06	5.66E+06	5.91E+04	6.06E+02	6.44E+01	1.16E+06
Variance	1.02E+11	1.58E+08	1.10E+12	1.70E+12	7.96E+12	1.81E+09	1.90E+05	2.42E+03	4.66E+12
Total									
Count	30	30	30	30	30	30	30	30	
Sum	6.07E+06	3.39E+05	3.25E+07	4.36E+07	1.30E+08	1.45E+06	1.14E+04	1.53E+03	
Average	2.02E+05	1.13E+04	1.08E+06	1.45E+06	4.32E+06	4.84E+04	3.80E+02	5.11E+01	
Variance	5.56E+10	9.93E+07	1.52E+12	1.27E+12	6.79E+12	1.54E+09	1.27E+05	2.19E+03	
ANOVA									
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*			
Sample	1.31E+13	2	6.54E+12	6.00	2.91E-03	3.04			
Columns	4.71E+14	7	6.72E+13	61.67	4.34E-48	2.05			
Interaction	3.09E+13	14	2.21E+12	2.03	1.71E-02	1.74			
Within	2.35E+14	216	1.09E+12						
Total	7.50E+14	239							

Table 8.7. ANOVA (two way with replicates) for seven elemental plasma (venous blood) concentrations ($ng.mL^{-1}$) obtained by ICP-MS from ten participants who participated in the physical stress study

	Li	Mg	P	K	Fe	Cu	Zn	Total
Phase A								
Count	7	7	7	7	7	7	7	49
Sum	2.80E+04	9.04E+04	6.64E+05	9.42E+05	5.55E+03	4.78E+03	6.23E+03	1.74E+06
Average	4.00E+03	1.29E+04	9.49E+04	1.35E+05	7.93E+02	6.83E+02	8.91E+02	3.55E+04
Variance	1.86E+06	1.90E+06	2.47E+08	7.68E+08	8.04E+04	2.12E+04	7.57E+04	2.82E+09
Phase C								
Count	7	7	7	7	7	7	7	49
Sum	2.60E+04	8.75E+04	6.05E+05	8.36E+05	5.63E+03	4.30E+03	5.55E+03	1.57E+06
Average	3.71E+03	1.25E+04	8.65E+04	1.19E+05	8.05E+02	6.14E+02	7.93E+02	3.20E+04
Variance	2.40E+06	1.95E+06	3.12E+08	1.14E+09	1.33E+04	2.25E+04	7.09E+04	2.33E+09
Phase D								
Count	7	7	7	7	7	7	7	49
Sum	2.61E+04	8.63E+04	6.05E+05	1.00E+06	5.84E+03	4.54E+03	5.70E+03	1.74E+06
Average	3.72E+03	1.23E+04	8.64E+04	1.43E+05	8.35E+02	6.49E+02	8.14E+02	3.54E+04
Variance	2.98E+06	1.48E+06	3.32E+08	6.84E+08	6.89E+04	2.06E+04	5.60E+04	2.95E+09
Total								
Count	21	21	21	21	21	21	21	
Sum	8.01E+04	2.64E+05	1.87E+06	2.78E+06	1.70E+04	1.36E+04	1.75E+04	
Average	3.81E+03	1.26E+04	8.92E+04	1.32E+05	8.11E+02	6.49E+02	8.33E+02	
Variance	2.19E+06	1.66E+06	2.84E+08	8.79E+08	4.91E+04	2.01E+04	6.26E+04	
ANOVA								
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*		
Sample	3.83E+08	2	1.91E+08	1.15	3.20E-01	3.07		
Columns	3.66E+11	6	6.09E+10	366	3.65E-77	2.17		
Interaction	1.97E+09	12	1.65E+08	0.99	4.65E-01	1.83		
Within	2.10E+10	126	1.67E+08					
Total	3.89E+11	146						

Table 8.8. Heart rate t-test scores (P). Values in bold are those where H0 was rejected (P < 0.05) indicating statistically significant differences between the indicating phases

Between phases	CZ1	CZ2	CZ3	CZ4	CZ5	CZ6	CZ7	CZ8	CZ9	CZ10	H_0 (✓)	H_0 (X)
A & B	**0.0000**	**0.0001**	**0.0000**	**0.0000**	**0.0002**	**0.0000**	**0.0001**	**0.0003**	**0.0035**	**0.0000**	0	10
B & C	**0.0001**	**0.0004**	**0.0000**	**0.0000**	**0.0010**	**0.0001**	**0.0003**	**0.0075**	0.0585	**0.0002**	1	9
C & D	0.0670	0.0570	**0.0314**	0.0556	0.0611	0.0790	0.1207	**0.0326**	**0.0079**	0.0585	7	3
A & D	**0.0000**	**0.0008**	**0.0004**	**0.0001**	**0.0019**	**0.0000**	**0.0000**	**0.0000**	0.4093	**0.0001**	1	9

Appendix III

Table 8.9. Calibration data for the nine statistically significant elements

Element	Gradient	Intercept	R^2	Standard Error	LOD ($ng.mL^{-1}$)	LOQ ($ng.mL^{-1}$)
Na	804864	14150099	0.9998	696332	2.60	8.65
Mg	27762	27690	0.9999	11071	1.20	3.99
Si	15180	171394	0.9988	20863	4.12	13.74
P	1690	8456	0.9993	1755	3.11	10.38
Mn	46667	1195	1.0000	1587	0.10	0.34
Fe	41869	6796	1.0000	3022	0.22	0.72
As	113864	19782	0.9997	28200	0.74	2.48
Sr	1297070	0	0.9999	21990	0.05	0.17
I	374	34043	0.9997	427	3.42	11.41

The calibration standards data in Table 8.9 shows that the nine elements of interest produced good linearity. As a result, concentration could be calculated using the equation of a straight line (y = mx + C). y = sample intensity obtained from the ICP-MS, m = gradient, x = unknown and C = intercept. Element concentrations were checked against the calculated limited of detection (LOD) and limit of quantification (LOQ) to ensure they were higher in concentration. If concentrations were lower than the differences between pre and post intervention samples could not be calculated for the selected element. All sample concentrations for the selected nine elements were higher than the LOD.

Table 8.10. Analysis of variance (ANOVA) data for pre and post intervention saliva

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Two-Factor Without Replication						
Pre (n=3)						
Rows	1.28E+10	2	6.38E+09	1.624	0.207	3.183
Columns	3.47E+12	25	1.39E+11	**35.332**	**0.000**	1.727
Error	1.96E+11	50	3.93E+09			
Total	3.68E+12	77				
Post (n=3)						
Rows	7.80E+08	2	3.90E+08	1.340	0.271	3.183
Columns	2.01E+12	25	8.04E+10	**276.033**	**0.000**	1.727
Error	1.46E+10	50	2.91E+08			
Total	2.03E+12	77				
Two-Factor With Replication						
Sample	1.61E+10	1	1.61E+10	**7.481**	**0.007**	3.932
Columns	5.35E+12	25	2.14E+11	**99.211**	**0.000**	1.612
Interaction	1.26E+11	25	5.02E+09	**2.328**	**0.002**	1.612
Within	2.25E+11	104	2.16E+09			
Total	5.72E+12	155				

The two-factor without replication ANOVA carried out shows that no statistically significant difference (95%) was evident between the replicate samples (rows) in the pre and post intervention samples as the critical F value (3.183) is bigger than the calculated F value (Pre F_{calc} = 1.624, Post F_{calc} = 1.340). Within each replicate, there is statistical evidence to suggest there is a difference between elements (columns) as for both the pre and post intervention samples the critical F value (1.727) is smaller than the calculated F values (Pre F_{calc} = 35.332, Post F_{calc} = 276.033).

Two way-factor with replication ANOVA shows the results of the relationship between the pre and post intervention samples. There is statistical evidence (95%) that elemental concentrations between the two groups (interaction) are different as the critical value of F (1.612) is smaller than the calculated F value (2.328).

Table 8.11. Descriptive statistics for all analysed elements showing F_{calc} and T_{calc} (P) scores

Sample	Pre		Post		F_{calc}	T_{calc} (P)
	μ	σ (n=3)	μ	σ (n=3)		
Li	1	1.77	2	1.41	0.78	0.68
B	55	14.67	36	7.28	0.40	0.13
Na	**120276**	**31755.22**	**52**	**14.34**	**0.00**	**0.00**
Mg	**3807**	**771.97**	**7318**	**895.17**	**0.85**	**0.01**
Al	41	26.24	115	163.98	0.05	0.48
Si	**340**	**32.09**	**541**	**70.20**	**0.35**	**0.01**
P	**214235**	**29940.89**	**151849**	**16516.24**	**0.47**	**0.03**
S	70385	6202.47	74974	7192.34	0.85	0.45
K	1087388	319364.24	829161	85644.33	0.13	0.25
Ca	39811	4514.35	41501	3453.74	0.74	0.63
V	0	0.05	0	0.00	0.00	0.37
Cr	1	0.19	1	0.14	0.66	0.13
Mn	**44**	**16.15**	**12**	**1.77**	**0.02**	**0.03**
Fe	**105**	**35.84**	**37**	**5.24**	**0.04**	**0.03**
Co	1	0.83	0	0.14	0.06	0.14
Ni	5	1.61	4	1.57	0.97	0.47
Cu	37	9.82	26	3.88	0.27	0.14
Zn	181	65.49	136	76.30	0.85	0.48
As	**3**	**1.34**	**7**	**1.34**	**1.00**	**0.01**
Se	1	1.08	2	0.32	0.16	0.64
Sr	**10**	**2.83**	**46**	**4.73**	**0.53**	**0.00**
Mo	1	1.07	1	0.73	0.63	0.89
In	0	0.00	0	0.00	0.74	0.86
Sn	2125	868.53	1159	150.55	0.06	0.13
I	**107567**	**25058.21**	**10363**	**560.42**	**0.00**	**0.00**
Pb	1	0.56	1	0.40	0.68	0.51

For the twenty six elements analysed an F test to test for variance, between the pre and post intervention samples, was conducted. For all elements the calculated F values were lower than the critical F values (F_{crit} = 23 degrees of freedom 2,2). As a result an equal variance T test (two tailed), to test differences in two experimental means, was carried out. T_{calc} values below 0.05 implied statistical difference at 95% between pre and post intervention samples.

Appendix IV

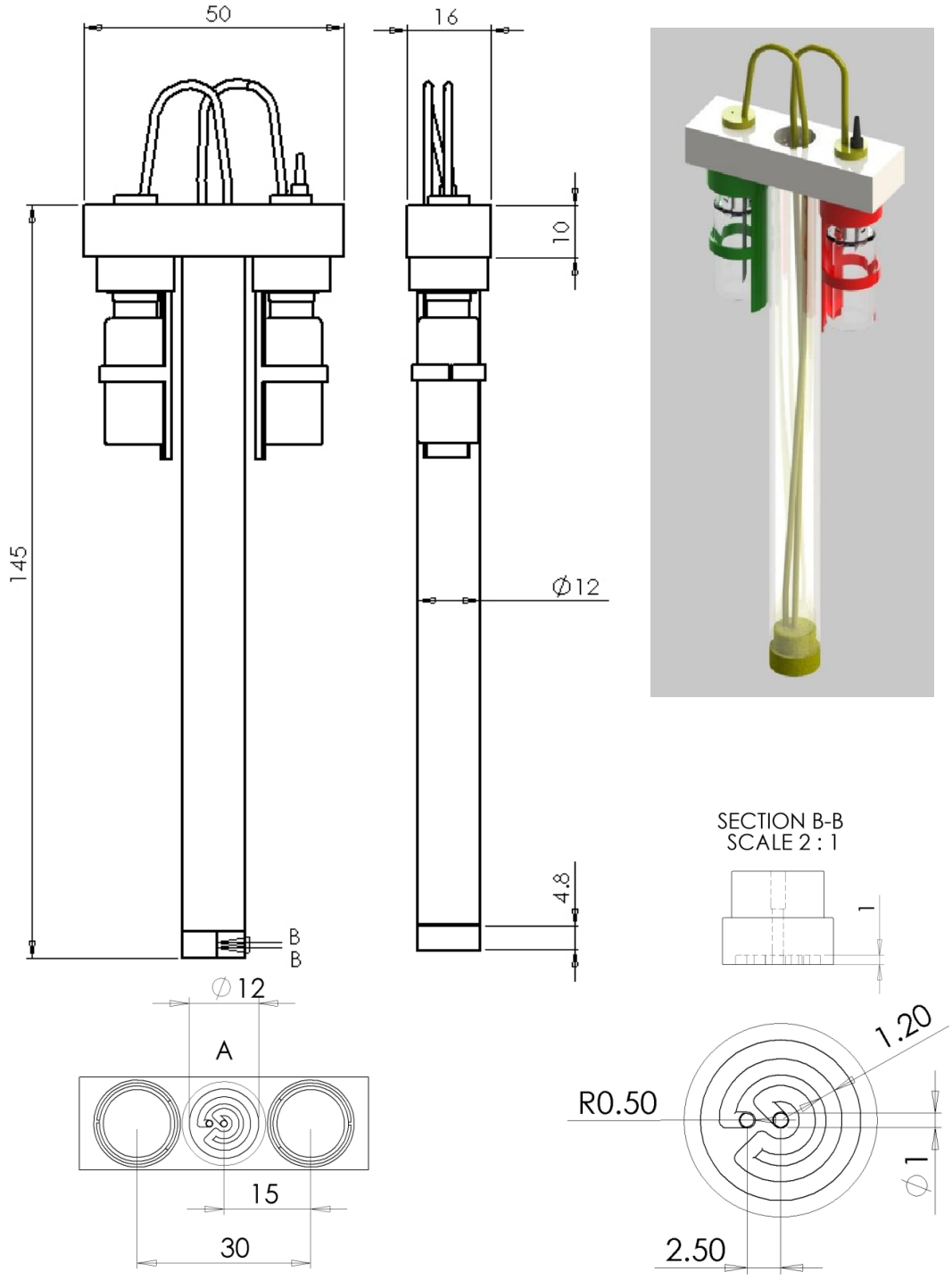


Figure 8.1. Schematic of the LSP, all dimensions in mm

Figure 8.2. Dilution mass (g) of samples collected using the LSP with 2% HNO_3 prior to ICP-MS anlysis. n = 3

Sample	2% HNO_3			Sample			Total
	μ	σ	RSD (%)	μ	σ	RSD (%)	
EDTA							
LSP Blank	3.9908	0.0001	0.0014	1.0400	0.0001	0.0056	5.03
B	4.0760	0.0001	0.0025	1.0418	0.0001	0.0096	5.12
B1	4.1263	0.0001	0.0014	1.0437	0.0001	0.0111	5.17
B2	4.0497	0.0001	0.0029	1.0471	0.0002	0.0146	5.10
B3	4.0820	0.0001	0.0014	1.0449	0.0002	0.0146	5.13
2% HNO_3							
LSP Blank	4.0940	0.0002	0.0037	1.0213	0.0002	0.0150	5.12
B	4.0382	0.0001	0.0029	0.9993	0.0001	0.0116	5.04
B1	3.9996	0.0001	0.0025	1.0305	0.0001	0.0056	5.03
B2	4.0283	0.0000	0.0000	1.0364	0.0001	0.0056	5.06
B3	3.9848	0.0000	0.0000	1.0377	0.0001	0.0096	5.02
DI H_2O							
LSP Blank	4.0352	0.0001	0.0029	1.0189	0.0001	0.0113	5.05
B	4.0714	0.0002	0.0038	1.0124	0.0001	0.0057	5.08
B1	4.0206	0.0001	0.0014	1.0184	0.0001	0.0057	5.04
B2	3.9692	0.0001	0.0029	1.0077	0.0001	0.0099	4.98
B3	3.9922	0.0001	0.0029	1.0071	0.0001	0.0115	5.00

Table 8.12. ANOVA: Two-factor with replication (n = 10) of the elements detected from the elemental profiling of the volar (AE) and plantar (BF) using the LSP

	Al	Si	P	S	K	Fe	Ni	Zn	Total
Volar									
Count	10	10	10	10	10	10	10	10	80
Sum	939	17009	242	6255	2786	743	62	214	28249
Average	94	1701	24	625	279	74	6	21	353
Variance	1946	988214	1094	480602	14302	3423	35	433	470980
Plantar									
Count	10	10	10	10	10	10	10	10	80
Sum	1027	21613	217	3579	7361	410	75	149	34431
Average	103	2161	22	358	736	41	7	15	430
Variance	2510	1021331	374	42877	878092	214	69	178	711820
Total									
Count	20	20	20	20	20	20	20	20	
Sum	1967	38622	458	9834	10147	1152	137	363	
Average	98	1931	23	492	507	58	7	18	
Variance	2131	1007675	697	266808	477803	2014	50	301	
ANOVA									
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*			
Sample	238798	1	238798	1.11	0	3.91			
Columns	60287921	7	8612560	**40.11**	0	2.07			
Interaction	2232053	7	318865	1.48	0	2.07			
Within	30921240	144	214731						
Total	93680012	159							

Table 8.13. Single factor ANOVA between different solvent used for the comparison of solvents experiment using the LSP

Groups	Count	Sum	Average	Variance	Source of Variation	SS	df	MS	F	P-value	F crit
EDTA Sample	3	1454804	484935	253490587	*Between Groups*	2.2E+11	2	1.1E+11	346.02	6.4E-07	5.14
HNO3 Sample	3	2056624	685541	544748674	*Within Groups*	1.9E+09	6	3.2E+08			
DI H2O Sample	3	913276	304425	147117936	*Total*	2.2E+11	8				

Table 8.14. ANOVA: Two-factor with replication (n = 10) of the elements detected from the four skin sites sampled using the LSP

	Mg	Al	S	K	Fe	Cu	Total
Axilla							
Count	10	10	10	10	10	10	60
Sum	5.47	2.23	11.78	23.72	7.88	4.87	55.95
Average	0.55	0.22	1.18	2.37	0.79	0.49	0.93
Variance	0.45	0.50	1.04	0.76	0.56	0.31	1.06
Dorsal							
Count	10	10	10	10	10	10	60
Sum	9.96	6.70	14.88	30.64	9.06	2.04	73.28
Average	1.00	0.67	1.49	3.06	0.91	0.20	1.22
Variance	0.36	0.55	0.66	0.25	0.41	0.11	1.20
Plantar							
Count	10	10	10	10	10	10	60
Sum	13.22	4.18	16.44	30.89	10.98	2.65	78.36
Average	1.32	0.42	1.64	3.09	1.10	0.27	1.31
Variance	0.55	0.50	1.57	0.24	0.21	0.12	1.37
Volar							
Count	10	10	10	10	10	10	60
Sum	3.79	8.69	10.26	26.85	7.35	2.27	59.20
Average	0.38	0.87	1.03	2.69	0.73	0.23	0.99
Variance	0.48	0.85	1.00	0.30	0.52	0.16	1.17
Total							
Count	40	40	40	40	40	40	
Sum	32.44	21.79	53.36	112.11	35.27	11.83	
Average	0.81	0.54	1.33	2.80	0.88	0.30	
Variance	0.57	0.62	1.05	0.44	0.41	0.18	
ANOVA							
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	
Sample	5.85	3	1.95	**3.75**	0.01	2.65	
Columns	161.57	5	32.31	**62.19**	0.00	2.26	
Interaction	9.24	15	0.62	1.19	0.28	1.71	
Within	112.24	216	0.52				
Total	288.90	239					

Table 8.15. ANOVA (two way with replicates) results for ions obtained from ESI-MS in negative ion mode for the profiling of the doral and plantar (BF)

Ion m/z	*Dorsal*				*Plantar*				*Total*			
	Count	Sum	Average	Variance	Count	Sum	Average	Variance	Count	Sum	Average	Variance
61.9884	9	14.52	1.61	2.35	9	17.61	1.96	2.20	18	32.13	1.79	2.17
72.0091	9	8.72	0.97	2.16	9	31.39	3.49	0.16	18	40.10	2.23	2.77
74.0248	9	24.57	2.73	0.44	9	33.40	3.71	0.04	18	57.97	3.22	0.48
82.0299	9	12.85	1.43	1.30	9	28.96	3.22	0.05	18	41.81	2.32	1.48
84.0456	9	1.43	0.16	0.23	9	21.06	2.34	0.31	18	22.50	1.25	1.51
87.0088	9	13.05	1.45	3.22	9	18.21	2.02	3.77	18	31.25	1.74	3.38
89.0245	9	37.40	4.16	2.49	9	43.80	4.87	0.03	18	81.20	4.51	1.32
90.0278	9	26.34	2.93	1.30	9	31.58	3.51	0.05	18	57.92	3.22	0.73
91.0221	9	20.59	2.29	0.87	9	25.80	2.87	0.03	18	46.39	2.58	0.51
92.9281	9	23.31	2.59	1.55	9	32.25	3.58	0.22	18	55.56	3.09	1.10
93.0346	9	35.78	3.98	0.05	9	40.69	4.52	0.04	18	76.47	4.25	0.12
94.0492	9	22.53	2.50	0.15	9	29.39	3.27	0.08	18	51.93	2.88	0.26
101.0233	9	1.18	0.13	0.15	9	0.00	0.00	0.00	18	1.18	0.07	0.08
104.0354	9	33.75	3.75	0.10	9	40.51	4.50	0.03	18	74.26	4.13	0.21
114.0215	9	15.68	1.74	1.97	9	28.32	3.15	0.02	18	44.00	2.44	1.46
114.0561	9	8.47	0.94	2.01	9	16.07	1.79	2.88	18	24.54	1.36	2.49
115.0185	9	27.18	3.02	1.42	9	34.61	3.85	0.02	18	61.79	3.43	0.86
116.0278	9	2.19	0.24	0.53	9	18.85	2.09	1.52	18	21.04	1.17	1.87
116.0506	9	24.77	2.75	0.18	9	33.32	3.70	0.03	18	58.09	3.23	0.34
118.0511	9	26.78	2.98	0.17	9	34.31	3.81	0.03	18	61.10	3.39	0.28
124.0075	9	2.20	0.24	0.54	9	11.72	1.30	1.77	18	13.92	0.77	1.38
128.0354	9	36.70	4.08	0.07	9	43.07	4.79	0.03	18	79.76	4.43	0.18
129.0389	9	21.12	2.35	2.07	9	32.87	3.65	0.02	18	53.98	3.00	1.43
130.0875	9	28.33	3.15	0.06	9	36.25	4.03	0.03	18	64.58	3.59	0.25
131.0828	9	18.83	2.09	1.70	9	23.49	2.61	2.27	18	42.32	2.35	1.94
132.0304	9	12.57	1.40	1.79	9	33.50	3.72	0.03	18	46.08	2.56	2.29
133.0316	9	10.72	1.19	2.13	9	18.62	2.07	2.43	18	29.34	1.63	2.35

137.0358	9	32.75	3.64	0.06	9	37.16	4.13	0.06	18	69.91	3.88	0.12
138.0392	9	20.85	2.32	0.13	9	26.24	2.92	0.09	18	47.09	2.62	0.20
146.0461	9	7.09	0.79	0.85	9	23.58	2.62	0.09	18	30.67	1.70	1.33
146.9833	9	22.94	2.55	1.09	9	28.38	3.15	0.13	18	51.33	2.85	0.67
148.9803	9	20.04	2.23	0.76	9	24.08	2.68	0.09	18	44.12	2.45	0.45
150.0175	9	7.27	0.81	0.94	9	21.51	2.39	0.05	18	28.78	1.60	1.13
151.0403	9	-50.00	-5.56	277.78	9	23.72	2.64	0.04	18	-26.28	-1.46	148.49
154.0625	9	15.31	1.70	0.56	9	26.25	2.92	0.10	18	41.56	2.31	0.70
Total	315	587.79	1.87	10.89	315	970.58	3.08	1.51				

ANOVA

Source of Variation	Sample	Columns	Interaction	Within	Total
SS	232.59	960.40	279.86	2655.38	4128.22
df	1	34	34	560	629
MS	232.59	28.25	8.23	4.74	
F	**49.05**	**5.96**	1.74		
P-value	7.2E-12	2.3E-21	0.0068		
F crit	3.86	1.45	1.45		

Table 8.16. ESI-MS negative ion mode results for t test between gender plantar (BF) samples for the profiling of the doral and plantar (BF). M BF = male plantar, F BF = female plantar. Samples ordered by largest to smallest difference between genders

Ion (m/z)	Male BF		Female BF		μ (M BF) - μ (F BF)	F_{calc}	$F_{4,3\ crit}$ (P = 0.05, 2 tail)	H_0 accepted ($F_{calc}<F_{crit}$)	P_{calc}	H_0 accepted ($P_{calc}>P_{crit}$)		
	μ (n=9)	σ (n=9)	μ (n=9)	σ (n=9)						P_{crit} (0.05)	P_{crit} (0.01)	P_{crit} (0.001)
128.0354	76928	34847	50582	5614	26346	0.052	9.12	No	0.16	✓	✓	✓
93.0346	47001	28568	25549	4447	21452	0.114	9.12	No	0.12	✓	✓	✓
89.0245	88066	34826	67197	24192	20870	0.748	9.12	No	0.37	✓	✓	✓
104.0354	39354	18325	27114	4158	12241	0.121	9.12	No	0.23	✓	✓	✓
137.0358	20652	14621	9909	1973	10743	0.110	9.12	No	0.11	✓	✓	✓
87.0088	5133	5042	880	1761	4253	0.891	9.12	No	0.13	✓	✓	✓
118.0511	8387	4097	5401	815	2986	0.083	9.12	No	0.21	✓	✓	✓
92.9281	7108	5682	4139	3431	2969	0.587	9.12	No	0.42	✓	✓	✓
115.0185	8461	2112	5854	1339	2607	0.897	9.12	No	0.07	✓	✓	✓
132.0304	6679	2477	4366	1021	2313	0.320	9.12	No	0.14	✓	✓	✓
130.0875	12353	6598	10213	1500	2140	0.068	9.12	No	0.72	✓	✓	✓
129.0389	5686	1842	3564	767	2123	0.565	9.12	No	0.04	✗	✓	✓
94.0492	3134	2345	1280	393	1854	0.315	9.12	No	0.08	✓	✓	✓
131.0828	2913	3036	1413	1195	1500	0.911	9.12	No	0.77	✓	✓	✓
74.0248	6177	3750	4950	1242	1227	0.196	9.12	No	0.71	✓	✓	✓
90.0278	4191	2277	3014	1435	1177	0.619	9.12	No	0.48	✓	✓	✓
116.0506	5963	3748	4930	582	1034	0.024	9.12	No	0.86	✓	✓	✓
82.0299	2312	1475	1321	292	992	0.182	9.12	No	0.18	✓	✓	✓
72.0091	4428	3742	3589	785	839	0.020	9.12	No	0.70	✓	✓	✓
138.0392	1409	1167	603	181	806	0.149	9.12	No	0.17	✓	✓	✓
146.9833	2113	1256	1420	846	693	0.547	9.12	No	0.62	✓	✓	✓
116.0278	816	596	161	186	655	0.044	9.12	No	0.13	✓	✓	✓
133.0316	1201	929	577	667	624	0.661	9.12	No	0.41	✓	✓	✓
61.9884	922	787	356	496	566	0.720	9.12	No	0.36	✓	✓	✓
84.0456	628	677	171	124	458	0.776	9.12	No	0.23	✓	✓	✓
114.0215	1655	576	1241	324	415	0.703	9.12	No	0.23	✓	✓	✓

154.0625	1229	1041	832	518	397	0.820	9.12	No	0.58	✓	✓	✓
124.0075	454	803	99	184	355	0.794	9.12	No	0.54	✓	✓	✓
146.0461	668	408	319	146	349	0.490	9.12	No	0.17	✓	✓	✓
91.0221	897	316	648	217	250	0.960	9.12	No	0.25	✓	✓	✓
148.9803	661	398	452	193	209	0.270	9.12	No	0.65	✓	✓	✓
150.0175	317	203	230	72	87	0.445	9.12	No	0.57	✓	✓	✓
151.0403	501	281	435	146	66	0.403	9.12	No	0.85	✓	✓	✓
114.0561	986	1354	956	694	30	0.798	9.12	No	0.42	✓	✓	✓
									H_0 accepted	33	34	34
									H_0 rejected	**1**	**0**	**0**
									Total	34	34	34

Table 8.17. ESI-MS negative ion mode results for t test between gender dorsal (TF) samples for the profiling of the doral (TF) and plantar (BF). M TF = male dorsal, F TF = female dorsal Samples ordered by largest to smallest difference between gender

Ion (m/z)	M TF		F TF		[μ (M TF) - μ (F TF)]	F_{calc}	$F_{4,3\ crit}$ (P = 0.05, 2 tail)	H_0 accepted ($F_{calc}<F_{crit}$)	P_{calc}	H_0 accepted ($P_{calc}>P_{crit}$)		
	μ (n=9)	σ (n=9)	μ (n=9)	σ (n=9)						P_{crit} (0.05)	P_{crit} (0.01)	P_{crit} (0.001)
89.0245	66065	43716	28978	24224	37087	0.001	9.12	No	0.32	✓	✓	✓
128.0354	15884	8458	11177	5088	4707	0.951	9.12	No	0.45	✓	✓	✓
104.0354	8344	5387	5147	2509	3197	0.901	9.12	No	0.40	✓	✓	✓
115.0185	4294	4038	1661	1698	2633	0.010	9.12	No	0.27	✓	✓	✓
90.0278	3127	2701	1163	1023	1965	0.010	9.12	No	0.28	✓	✓	✓
92.9281	2438	1574	570	640	1868	0.573	9.12	No	0.33	✓	✓	✓
146.9833	1499	836	276	260	1223	0.023	9.12	No	0.15	✓	✓	✓
131.0828	1164	1832	371	410	794	0.970	9.12	No	0.60	✓	✓	✓
87.0088	2142	3620	1362	2724	780	0.930	9.12	No	0.48	✓	✓	✓
74.0248	798	960	1574	1425	776	0.786	9.12	No	0.37	✓	✓	✓
129.0389	1145	1467	1577	1408	432	0.769	9.12	No	0.85	✓	✓	✓
118.0511	1444	938	1023	612	421	0.482	9.12	No	0.53	✓	✓	✓
133.0316	532	791	132	217	400	0.729	9.12	No	0.92	✓	✓	✓
114.0215	681	968	301	352	381	0.711	9.12	No	0.55	✓	✓	✓
137.0358	5067	2233	4693	2308	374	0.290	9.12	No	0.71	✓	✓	✓
61.9884	611	384	272	544	339	0.736	9.12	No	0.16	✓	✓	✓
148.9803	477	231	158	123	320	0.013	9.12	No	0.22	✓	✓	✓
91.0221	566	359	289	384	278	0.006	9.12	No	0.24	✓	✓	✓
130.0875	1702	1064	1497	810	205	0.697	9.12	No	0.74	✓	✓	✓
132.0304	292	292	103	133	190	0.902	9.12	No	0.64	✓	✓	✓
116.0506	776	786	891	737	115	0.539	9.12	No	0.95	✓	✓	✓
72.0091	309	691	403	653	94	0.781	9.12	No	0.50	✓	✓	✓
114.0561	206	460	298	404	92	0.738	9.12	No	0.47	✓	✓	✓
82.0299	180	229	96	132	84	0.724	9.12	No	0.97	✓	✓	✓
138.0392	300	239	235	142	65	0.689	9.12	No	0.70	✓	✓	✓
146.0461	62	74	2	3	60	0.118	9.12	No	0.16	✓	✓	✓

93.0346	10444	3873	10483	5065	39	0.267	9.12	No	0.84	✓	✓	✓
124.0075	32	71	0	0	32	n / a	9.12	No	n / a	✓	✓	✓
116.0278	31	69	0	0	31	n / a	9.12	No	n / a	✓	✓	✓
150.0175	27	59	43	38	16	0.931	9.12	No	0.20	✓	✓	✓
94.0492	399	227	413	249	15	0.131	9.12	No	0.76	✓	✓	✓
154.0625	102	96	117	117	14	0.918	9.12	No	0.87	✓	✓	✓
84.0456	5	12	0	0	5	n / a	9.12	No	n / a	✓	✓	✓
151.0403	0	0	0	0	0	n / a	9.12	No	n / a	✓	✓	✓
									H_0 accepted	34	34	34
									H_0 rejected	**0**	**0**	**0**
									Total	34	34	34

Table 8.18. ANOVA (two way with replicates) results for ions obtained from ESI-MS in positive ion mode for the profiling of the doral and plantar (BF)

Ion m/z	*Dorsal*				*Plantar*				*Total*			
	Count	Sum	Average	Variance	Count	Sum	Average	Variance	Count	Sum	Average	Variance
56.9784	9	5.82	0.65	1.70	9	5.95	0.66	1.79	18	11.78	0.65	1.64
60.0578	9	13.74	1.53	2.20	9	26.07	2.90	1.39	18	39.81	2.21	2.19
70.0783	9	19.80	2.20	1.70	9	34.47	3.83	0.25	18	54.27	3.02	1.62
71.0822	9	5.04	0.56	1.24	9	11.91	1.32	2.61	18	16.94	0.94	1.97
78.9706	9	21.73	2.41	2.09	9	23.88	2.65	2.34	18	45.61	2.53	2.10
82.0282	9	5.06	0.56	1.30	9	5.47	0.61	1.46	18	10.53	0.59	1.30
83.0233	9	8.97	1.00	2.24	9	18.10	2.01	2.34	18	27.07	1.50	2.43
84.0955	9	14.07	1.56	1.67	9	30.27	3.36	0.17	18	44.34	2.46	1.72
90.1047	9	14.51	1.61	1.72	9	29.54	3.28	0.06	18	44.05	2.45	1.58
93.0590	9	28.28	3.14	0.08	9	27.03	3.00	1.40	18	55.31	3.07	0.70
94.9435	9	27.94	3.10	1.56	9	35.09	3.90	0.09	18	63.04	3.50	0.94
96.9377	9	25.77	2.86	1.26	9	33.07	3.67	0.05	18	58.84	3.27	0.79
98.9343	9	19.82	2.20	1.60	9	28.80	3.20	0.04	18	48.61	2.70	1.03
99.0004	9	14.66	1.63	3.84	9	22.79	2.53	2.28	18	37.45	2.08	3.10
104.1145	9	20.18	2.24	1.17	9	32.12	3.57	0.04	18	52.29	2.91	1.04
110.0838	9	23.96	2.66	2.50	9	35.55	3.95	0.32	18	59.51	3.31	1.77
110.9912	9	8.58	0.95	2.06	9	23.86	2.65	1.13	18	32.44	1.80	2.26
112.9112	9	33.86	3.76	0.38	9	40.52	4.50	0.18	18	74.38	4.13	0.41
114.0034	9	15.96	1.77	2.96	9	31.14	3.46	0.29	18	47.10	2.62	2.28
114.9070	9	27.48	3.05	1.69	9	37.86	4.21	0.14	18	65.34	3.63	1.21
115.1008	9	24.30	2.70	1.15	9	36.03	4.00	0.09	18	60.33	3.35	1.03
116.0842	9	18.64	2.07	2.65	9	40.07	4.45	0.26	18	58.71	3.26	2.87
120.0073	9	36.54	4.06	0.09	9	41.11	4.57	0.04	18	77.64	4.31	0.13
121.0520	9	36.94	4.10	0.09	9	37.61	4.18	0.19	18	74.55	4.14	0.14
122.9668	9	25.45	2.83	2.71	9	35.40	3.93	0.18	18	60.85	3.38	1.68
124.9750	9	18.55	2.06	2.44	9	27.73	3.08	0.24	18	46.29	2.57	1.53
130.0939	9	12.73	1.41	2.89	9	27.00	3.00	3.23	18	39.73	2.21	3.54
132.9958	9	29.17	3.24	0.19	9	31.95	3.55	0.09	18	61.12	3.40	0.16
133.1022	9	13.80	1.53	3.32	9	22.22	2.47	3.51	18	36.03	2.00	3.45
134.0272	9	28.82	3.20	1.58	9	35.30	3.92	0.09	18	64.12	3.56	0.92
135.9943	9	9.37	1.04	2.44	9	29.50	3.28	1.56	18	38.87	2.16	3.21
137.0760	9	8.60	0.96	2.08	9	33.48	3.72	0.11	18	42.08	2.34	3.05
138.0667	9	21.53	2.39	1.94	9	33.59	3.73	0.09	18	55.12	3.06	1.43

139.0653	9	33.21	3.69	0.19	9	39.15	4.35	0.12	18	72.36	4.02	0.26
139.9322	9	19.06	2.12	1.84	9	30.66	3.41	0.09	18	49.72	2.76	1.35
140.0849	9	23.24	2.58	0.99	9	33.85	3.76	0.18	18	57.08	3.17	0.91
141.9948	9	25.39	2.82	1.19	9	32.47	3.61	0.11	18	57.86	3.21	0.78
142.1064	9	4.65	0.52	1.09	9	17.89	1.99	2.31	18	22.54	1.25	2.17
144.9370	9	14.07	1.56	3.45	9	22.91	2.55	3.70	18	36.98	2.05	3.62
148.9789	9	17.20	1.91	2.17	9	30.02	3.34	0.10	18	47.23	2.62	1.61
150.0297	9	33.17	3.69	0.11	9	40.65	4.52	0.05	18	73.81	4.10	0.26
150.9935	9	24.85	2.76	2.76	9	31.90	3.54	1.85	18	56.76	3.15	2.33
154.0362	9	5.31	0.59	1.38	9	29.17	3.24	0.09	18	34.48	1.92	2.55
155.0909	9	5.82	0.65	1.67	9	32.46	3.61	0.32	18	38.28	2.13	3.25
156.9937	9	29.54	3.28	1.66	9	31.31	3.48	1.79	18	60.85	3.38	1.64
157.0029	9	13.54	1.50	3.33	9	26.55	2.95	1.55	18	40.09	2.23	2.85
160.0446	9	26.02	2.89	0.08	9	32.03	3.56	0.04	18	58.04	3.22	0.18
160.9231	9	12.59	1.40	2.81	9	16.40	1.82	3.02	18	28.99	1.61	2.79
162.0714	9	23.77	2.64	1.04	9	31.48	3.50	0.04	18	55.25	3.07	0.70
164.0496	9	24.08	2.68	0.11	9	31.71	3.52	0.06	18	55.79	3.10	0.27
164.1488	9	15.09	1.68	1.95	9	22.64	2.52	1.19	18	37.73	2.10	1.66
164.9579	9	3.78	0.42	1.59	9	16.45	1.83	3.20	18	20.22	1.12	2.78
165.0289	9	5.42	0.60	1.47	9	0.00	0.00	0.00	18	5.42	0.30	0.79
165.9969	9	9.77	1.09	2.65	9	32.43	3.60	0.18	18	42.20	2.34	3.01
166.9725	9	24.49	2.72	1.40	9	32.52	3.61	0.13	18	57.01	3.17	0.94
168.9755	9	3.42	0.38	1.30	9	14.48	1.61	2.36	18	17.90	0.99	2.12
172.0192	9	6.66	0.74	1.25	9	21.85	2.43	0.86	18	28.51	1.58	1.74
172.9783	9	6.08	0.68	1.82	9	17.79	1.98	2.23	18	23.87	1.33	2.35
173.0899	9	6.50	0.72	1.18	9	27.22	3.02	0.12	18	33.72	1.87	2.01
176.0920	9	20.59	2.29	0.76	9	25.80	2.87	1.18	18	46.39	2.58	1.00
177.0821	9	6	1	2	9	30	3	0	18	35.59	1.98	2.68
Total	549	1083	2	3	549	1716	3	2				

ANOVA

Source of Variation	Sample	Columns	Interaction	Within	Total
SS	366	951	149	1250	2716
df	1	60	60	976	1097
MS	366	15.9	2.49	1.28	
F	285	12.4	1.94		
P-value	2.41E-56	9.65E-84	3.94E-05		
F crit	3.85	1.33	1.33		

Table 8.19. ESI-MS positive ion mode results for t test between gender plantar (BF) samples for the profiling of the doral and plantar (BF). M BF = male plantar, F BF = female plantar. Samples ordered by largest to smallest difference between genders

Ion (m/z)	M BF (Total)		F BF (Total)		[μ (M BF) - μ (F TF)]	F_{calc}	$F_{3,3crit}$	H_0 accepted	P_{calc}	H_0 accepted (P_{calc}>P_{crit})		
	μ (n=9)	σ (n=9)	μ (n=9)	σ (n=9)						P_{crit} (0.05)	P_{crit} (0.01)	P_{crit} (0.001)
116.0842	61942	35301	21672	22736	40270	13.96	15.44	Yes	0.061	✓	✓	✓
112.9112	58136	30811	27252	23314	30884	10.56	15.44	Yes	0.143	✓	✓	✓
150.0297	44476	21291	26880	6863	17596	14.30	15.44	Yes	0.258	✓	✓	✓
114.9070	29077	15739	11862	9259	17215	10.96	15.44	Yes	0.093	✓	✓	✓
155.091	15776	21948	2193	1457	13584	2.52	15.44	Yes	0.125	✓	✓	✓
115.101	16398	7668	7076	3552	9322	15.51	15.44	Yes	0.068	✓	✓	✓
121.0520	25994	20817	16823	13587	9171	6.80	15.44	Yes	0.451	✓	✓	✓
110.0838	19252	16187	12062	14170	7189	2.76	15.44	Yes	0.553	✓	✓	✓
140.0849	12849	19100	6064	4162	6786	3.71	15.44	Yes	0.722	✓	✓	✓
139.0653	31209	19948	24485	19968	6723	17.55	15.44	Yes	0.435	✓	✓	✓
70.0783	14006	12848	7392	7927	6614	4.43	15.44	Yes	0.362	✓	✓	✓
114.0034	7619	4119	1556	1462	6063	7.47	15.44	Yes	0.024	×	✓	✓
130.0939	14331	18517	8912	16153	5419	10.64	15.44	Yes	0.136	✓	✓	✓
138.0667	8852	5730	4015	1787	4837	8.88	15.44	Yes	0.175	✓	✓	✓
165.9969	7803	4389	3243	3457	4560	9.47	15.44	Yes	0.101	✓	✓	✓
134.0272	12036	6561	7541	4824	4495	17.66	15.44	Yes	0.256	✓	✓	✓
137.0760	8388	4660	4237	2149	4151	7.81	15.44	Yes	0.263	✓	✓	✓
150.9935	12063	8602	8155	6051	3908	27.02	15.44	Yes	0.329	✓	✓	✓
99.0004	4091	3583	432	726	3659	14.56	15.44	Yes	0.027	×	✓	✓
156.994	10166	4843	7058	7254	3108	51.90	15.44	Yes	0.263	✓	✓	✓
144.937	3899	4999	6980	6815	3081	1.09	15.44	Yes	0.629	✓	✓	✓
135.9943	6190	3226	3276	2501	2914	41.47	15.44	Yes	0.251	✓	✓	✓
94.9435	10851	5371	8196	7568	2655	13.45	15.44	Yes	0.410	✓	✓	✓
164.9579	3130	3860	514	890	2616	1.45	15.44	Yes	0.557	✓	✓	✓
96.9377	6485	2673	3882	2115	2603	22.05	15.44	Yes	0.152	✓	✓	✓
148.9789	3930	2849	1429	356	2502	7.50	15.44	Yes	0.115	✓	✓	✓
166.9725	6482	4806	4195	2819	2287	5.62	15.44	Yes	0.601	✓	✓	✓
133.1022	5199	4786	2917	3521	2282	1.64	15.44	Yes	0.434	✓	✓	✓
122.9668	13000	7020	10764	11372	2237	5.03	15.44	Yes	0.653	✓	✓	✓
132.996	5390	3927	3209	1670	2182	7.86	15.44	Yes	0.452	✓	✓	✓

84.0955	4027	2557	2132	1837	1896	6.47	15.44	Yes	0.299	✓	✓	✓
164.0496	4635	2478	2754	645	1881	11.19	15.44	Yes	0.326	✓	✓	✓
154.036	2878	1365	1162	493	1716	13.84	15.44	Yes	0.053	✓	✓	✓
71.0822	1714	3091	62	122	1652	2.24	15.44	Yes	0.252	✓	✓	✓
164.1488	2087	3451	622	621	1464	2.22	15.44	Yes	0.787	✓	✓	✓
139.9322	3730	2281	2332	1703	1398	14.59	15.44	Yes	0.275	✓	✓	✓
104.1145	4634	2097	3327	1176	1308	19.98	15.44	Yes	0.387	✓	✓	✓
177.0821	2861	1175	1727	755	1134	19.38	15.44	Yes	0.180	✓	✓	✓
141.995	4573	2417	5522	3677	948	19.88	15.44	Yes	0.963	✓	✓	✓
93.0590	3261	3120	2429	1975	832	2.56	15.44	Yes	0.602	✓	✓	✓
60.0578	3398	4777	2622	4302	777	13.25	15.44	Yes	0.304	✓	✓	✓
142.106	1155	1088	409	382	746	1.75	15.44	Yes	0.967	✓	✓	✓
56.9784	665	1325	1	1	664	2.79	15.44	Yes	0.202	✓	✓	✓
120.0073	40310	18989	40954	20483	644	19.94	15.44	Yes	0.935	✓	✓	✓
98.9343	2005	919	1374	484	632	25.51	15.44	Yes	0.262	✓	✓	✓
160.9231	1469	1456	847	1262	622	1.02	15.44	Yes	0.724	✓	✓	✓
172.9783	927	701	375	450	553	1.47	15.44	Yes	0.361	✓	✓	✓
160.045	4203	2161	3676	1452	527	18.13	15.44	Yes	0.813	✓	✓	✓
168.9755	720	708	227	278	493	1.21	15.44	Yes	0.647	✓	✓	✓
176.0920	1816	860	1328	905	488	49.05	15.44	Yes	0.317	✓	✓	✓
83.0233	1016	951	625	742	392	1.51	15.44	Yes	0.880	✓	✓	✓
124.9750	1951	931	1599	1927	352	13.02	15.44	Yes	0.328	✓	✓	✓
78.9706	2336	2016	2648	2690	312	1.30	15.44	Yes	0.919	✓	✓	✓
90.1047	2310	1100	2054	1294	256	17.22	15.44	Yes	0.691	✓	✓	✓
82.0282	233	350	1	1	232	2.26	15.44	Yes	0.194	✓	✓	✓
157.0029	4084	6015	3892	5244	192	8.99	15.44	Yes	0.436	✓	✓	✓
172.019	460	132	600	515	140	125.36	15.44	Yes	0.463	✓	✓	✓
162.0714	3370	1591	3479	1477	110	20.38	15.44	Yes	0.835	✓	✓	✓
173.09	1430	1393	1350	905	80	7.20	15.44	Yes	0.932	✓	✓	✓
110.9912	1219	586	1190	1860	29	25.21	15.44	Yes	0.271	✓	✓	✓
165.0289	1	0	1	1	0	n / a	15.44	n / a	n / a	✓	✓	✓
									H_0 accepted	59	61	61
									H_0 rejected	2	0	0
									Total	61	61	61

Table 8.20. ESI-MS positive ion mode results for t test between gender dorsal (TF) samples for the profiling of the doral and plantar (BF). M TF = male dorsal, F TF = female dorsal. Samples ordered by largest to smallest difference between genders

Ion (m/z)	M TF (Total)		F TF (Total)		[μ (M TF) - μ (F TF)]	F_{calc}	$F_{3,3crit}$	H_0 accepted	P_{calc}	H_0 accepted (P_{calc}>P_{crit})		
	μ	σ (n = 9)	μ	σ (n = 9)						P_{crit} (0.05)	P_{crit} (0.01)	P_{crit} (0.001)
112.9112	23204	24119	2224	1047	20980	3.451	15.44	Yes	0.03	×	✓	✓
114.9070	11172	12801	728	638	10444	5.361	15.44	Yes	0.07	✓	✓	✓
122.9668	7659	7801	1904	2197	5756	15.739	15.44	Yes	0.09	✓	✓	✓
99.0004	5594	5783	1	1	5593	2.895	15.44	Yes	0.01	×	✓	✓
94.9435	6559	6100	1908	1959	4651	11.877	15.44	Yes	0.18	✓	✓	✓
156.994	7748	2217	3137	4389	4611	214.204	15.44	Yes	0.13	✓	✓	✓
150.9935	7304	9438	2817	3428	4488	7.999	15.44	Yes	0.16	✓	✓	✓
166.9725	4970	7824	522	539	4448	4.520	15.44	Yes	0.12	✓	✓	✓
150.0297	7863	6157	4207	2570	3656	10.340	15.44	Yes	0.27	✓	✓	✓
139.0653	8341	8496	5903	3091	2438	3.510	15.44	Yes	0.98	✓	✓	✓
114.0034	2301	1960	79	155	2222	1.525	15.44	Yes	0.07	✓	✓	✓
96.9377	2924	1715	725	585	2199	21.504	15.44	Yes	0.13	✓	✓	✓
134.0272	4049	2842	5904	6015	1855	22.002	15.44	Yes	0.50	✓	✓	✓
116.0842	2654	5303	1067	893	1587	1.348	15.44	Yes	0.69	✓	✓	✓
139.9322	1647	1569	129	147	1519	2.665	15.44	Yes	0.06	✓	✓	✓
132.996	2936	1093	1484	1074	1452	37.223	15.44	Yes	0.13	✓	✓	✓
130.0939	1566	2417	298	594	1269	1.376	15.44	Yes	0.34	✓	✓	✓
160.9231	1383	1657	122	241	1261	1.838	15.44	Yes	0.27	✓	✓	✓
120.0073	13451	8712	14696	10898	1245	10.970	15.44	Yes	0.93	✓	✓	✓
164.9579	1198	2677	1	1	1197	2.853	15.44	Yes	0.41	✓	✓	✓
110.0838	2952	5160	4145	2796	1193	1.499	15.44	Yes	0.83	✓	✓	✓
148.9789	1263	1225	105	208	1158	7.907	15.44	Yes	0.01	×	×	✓
164.1488	1333	2656	187	123	1146	3.354	15.44	Yes	0.42	✓	✓	✓
165.9969	1109	1068	1	1	1108	3.187	15.44	Yes	0.07	✓	✓	✓
104.1145	1181	1259	205	215	976	2.236	15.44	Yes	0.19	✓	✓	✓
124.9750	1370	1234	491	569	879	1.443	15.44	Yes	0.36	✓	✓	✓
135.9943	807	750	1	1	806	2.931	15.44	Yes	0.07	✓	✓	✓
98.9343	901	375	207	243	695	56.112	15.44	Yes	0.05	×	✓	✓
70.0783	1194	1942	509	449	686	1.046	15.44	Yes	0.84	✓	✓	✓
157.0029	2305	4292	1688	3374	617	1.085	15.44	Yes	0.46	✓	✓	✓

83.0233	607	598	1	1	606	2.693	15.44	Yes	0.07	✓	✓	✓
133.1022	1622	1800	1043	2085	579	1.062	15.44	Yes	0.39	✓	✓	✓
172.9783	566	999	1	1	565	2.823	15.44	Yes	0.20	✓	✓	✓
168.9755	530	1184	1	1	529	2.343	15.44	Yes	0.41	✓	✓	✓
144.937	1383	1966	1907	2778	524	1.108	15.44	Yes	0.80	✓	✓	✓
56.9784	512	988	1	1	511	2.641	15.44	Yes	0.20	✓	✓	✓
110.9912	497	587	1	1	496	2.490	15.44	Yes	0.07	✓	✓	✓
60.0578	618	543	156	311	462	1.149	15.44	Yes	0.14	✓	✓	✓
90.1047	537	533	87	173	450	3.175	15.44	Yes	0.03	×	✓	✓
138.0667	1451	1930	1045	1062	407	1.186	15.44	Yes	0.87	✓	✓	✓
115.101	1555	1990	1169	917	386	2.085	15.44	Yes	0.57	✓	✓	✓
137.0760	444	759	124	247	320	1.467	15.44	Yes	0.63	✓	✓	✓
78.9706	1654	1614	1335	1556	319	10.740	15.44	Yes	0.21	✓	✓	✓
165.0289	312	594	1	1	311	2.295	15.44	Yes	0.20	✓	✓	✓
140.0849	940	542	632	485	308	25.556	15.44	Yes	0.31	✓	✓	✓
84.0955	395	470	138	267	257	1.176	15.44	Yes	0.23	✓	✓	✓
164.0496	726	700	502	349	224	7.521	15.44	Yes	0.64	✓	✓	✓
82.0282	220	427	1	1	219	2.028	15.44	Yes	0.20	✓	✓	✓
155.091	309	689	109	216	200	1.170	15.44	Yes	0.98	✓	✓	✓
154.036	195	295	1	1	194	2.130	15.44	Yes	0.19	✓	✓	✓
162.0714	1027	833	853	414	174	1.915	15.44	Yes	0.57	✓	✓	✓
160.045	1010	679	845	575	165	14.608	15.44	Yes	0.63	✓	✓	✓
176.0920	418	183	273	213	145	60.220	15.44	Yes	0.26	✓	✓	✓
71.0822	145	223	1	1	144	1.919	15.44	Yes	0.19	✓	✓	✓
142.106	108	240	22	41	87	1.491	15.44	Yes	0.93	✓	✓	✓
121.0520	15563	12608	15645	8037	82	9.242	15.44	Yes	0.87	✓	✓	✓
93.0590	1644	1319	1717	878	73	10.760	15.44	Yes	0.84	✓	✓	✓
177.0821	194	431	124	247	70	1.018	15.44	Yes	0.93	✓	✓	✓
172.019	90	133	27	51	64	1.611	15.44	Yes	0.60	✓	✓	✓
173.09	40	87	65	74	25	1.408	15.44	Yes	0.45	✓	✓	✓
141.995	1560	1365	1554	877	6	2.138	15.44	Yes	0.52	✓	✓	✓
									H_0 accepted	56	60	61
									H_0 rejected	5	1	0
									Total	61	61	61

Appendix V Documentation for studies involving human volunteers

Study Participant Information Sheets

Chapter 2: A study into the effect of psychological stress on the elemental content of human forehead sweat as measured by ICP-MS

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 two separate visits each lasting about 2 hours. During this visit we will first ask you a series of questions relating to factors that can influence the chemical substances we are interested in. 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 dental and personal care products. We will also ask you for information about diet and digestion. For the same reasons, you may be asked to refrain from eating, drinking (other than unflavoured water), smoking and using personal care products for an agreed time period prior to the study visit.

At the beginning of the first session you will be asked to read and fill out a health screen questionnaire and be given the opportunity to ask any questions that you may have about the research. Then if you are still willing to participate you will be asked to sign a consent form.

Next you will be asked to fill in some short questionnaires on demographics, health habits, anxiety, mood and leadership. Once these are complete the investigator will measure your blood pressure and heart rate using an inflatable cuff attached to the upper part of your arm.

After we have collected this information, we will then begin taking samples. This involves placing a small section of an inert strip on the surface of your skin and covering it with a dressing. We will also take a breath sample; this involves wearing a face mask that is connected to an air supply. You will be asked to breathe normally throughout the test, whilst sitting comfortably in a chair. A sampling tube is connected to the mask on which we will collect the sample.

When you are ready you will be asked to perform four, computer based cognitive function tests which assess memory, executive functioning and attention. These tests require you to press buttons on the keyboard to answer the questions.

After we have collected this information, we will then begin taking saliva samples. This involves placing a small strip of an inert material under your , it will be held by thin locking forceps that you will have control of to prevent you from swallowing or choking on it. The forceps have been selected for your comfort and are completely rounded and should feel similar to brushing your teeth or using an oral thermometer. This sample will take up to a maximum of 5 minutes. Once sampling is complete you will simply hand the forceps to the investigator. We will then issue you with a collection tube for whole saliva sample collection. This involves sitting comfortably in a chair with your head tilted slightly forward. In this position saliva will begin pool at the front of your mouth. After 30 seconds to 1 minute a

sufficient amount of saliva will have collected, at this time you may dispense the pooled saliva into the collection tube. To help this procedure you may wish to imagine your favourite food. We will continue to collect saliva in this manner until you feel you wish to stop or a sufficient quantity of saliva has been collected.

At the end of the sampling period the investigator will take the collection tube from you. At this point tissues and drink of water will be available to you should you wish.

During the next ten minutes you will be asked to complete either a mental arithmetic task or relax and listen to some music, you will complete different tasks on each visit. The mental arithmetic test is known as the Paced Auditory Serial Addition Test (PASAT), this is designed to require inert mental effort, with incorrect responses eliciting a 'buzzer' sound that you may find stressful. You will be given a full explanation of the test prior to starting and will be given the opportunity to ask any questions that you may have. During the task your blood pressure and heart rate will be monitored.

The cognitive tests and chemical tests described above will then be repeated. We may ask for any or all of the described samples.

Chapter 3: The effect of physical stress on elemental content in human plasma, skin and urine

Ethical Advisory Committee

PART 1 Monday, 31st January – Friday 4th February 2011

Invitation to participant

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?

This study is investigating the effect of exercise on physiological functions combined with the chemical profile of the body. We are testing new methods of detecting changes in the human body by monitoring tiny amounts of chemical substances in saliva, breath, urine, blood and on the 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. In this study we aim to determine chemical markers for physical stress by taking measurements before and after a series of tests.

Why have I been invited to participate?

You have been invited because you are a healthy, non-smoking adult between the ages of 18 and 35.

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 will 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 approximately 3 hours. During this visit we will first ask you a series of questions relating to factors that can influence the chemical substances we are interested in.

Please see the brief information sheet for further detail.

At the beginning of the visit you will be asked to provide a urine sample and both your weight and height measurements will be taken. You will be asked to wear a heart monitor on your chest, and a skin conductivity meter on your upper left arm, throughout the session. Also, please be aware that you will be required to drink a small amount of water at pre-determined points during your visit.

There will be four 30 minute periods of sampling. The first, third and fourth period will be very similar in sampling whilst sitting on a chair within the chamber whereas in the second period, you will be asked to exercise on a cyclometer.

In the non-exercise periods the following samples will be taken;
Urine
2 x sweat samples by placing patches onto your forehead
Skin tremor
15ml of blood taken via a butterfly cannula from your left arm
Saliva (drool)
Saliva – PDMS rod
Breath
Breath (BioVoc) DMS – Only the third and fourth period
During the exercise period the following samples will be taken;
2 x sweat samples by placing patches onto your forehead
Breath (BioVoc) DMS – Only the third and fourth period

You will then have the option to take shower and if you want a shower – towel will be provided. After your shower you will be asked to complete a final questionnaire (very quick) and you will be provided with snacks and a sports drink (Powerade). Any questions you may have will be answered and you will then be free to leave the laboratory area /building.
*Please note that a new puncture will be made to your left arm for every blood sample taken. However, in some cases it may be appropriate to take blood out of the right arm.

What will I have to do?
You will have to refrain from smoking, eating, brushing your teeth, using mouthwash, taking throat lozenges or chewing gum etc. or drinking anything other than unflavoured water for 60 minutes prior to the study visit.
We also ask you not to swim, shower, use perfumed products (which include not only perfume, but also deodorants, after-shaves), any facial products (such as make-up or creams (scented or unscented) or wash your hair on the morning of your visit. You may shower the evening before.
It is important that you do not drink alcohol the night before your visit.
Please wear comfortable clothing (e.g. shorts and t-shirt) and appropriate footwear as you will be conducting part of the experiment on a cyclometer.

What are the possible disadvantages and risks of taking part?
We do not anticipant any risk from taking part in this study. New and sterile needles and cannulas will be used for every sample of blood that is taken.

There is some mild discomfort in providing a blood sample and a risk of bruising is always associated with giving a blood sample.
Following the experiment, you may feel a little fatigued. This is nothing to worry about as you should re-energise upon consuming some of the energy snacks /drinks.

What are the possible benefits of taking part?
There will be no direct benefits to you from taking part in this research. You will however receive information regarding your height and weight as well as your blood pressure and heart rate upon request.
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.

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 study at any stage with no adverse consequences.
We will ensure that you are able to stop the sampling procedure at any time quickly and easily should you want to.

What is there is a problem?
If you have any 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 01509 222 549 – Prof. Paul Thomas). 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 the 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 ground 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 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. All information will be treated in accordance to with the Data Protection Act 1998.

What will happen to the samples I give?
Two sets of skin samples are collected. The first set of skin samples are collected on inert strips which are placed directly into small tubes when removed from the skin; these are then

sealed and will be processed at the Department of Chemistry at Loughborough University. The second set of skin samples will be collected on cotton pads which are placed directly into polypropylene tubes when collected; these are then sealed and will be processed at the Department of Chemistry at Loughborough University.

The components are collected from exhaled breath onto adsorbent tubes which are sealed and will be processed at the Department of Chemistry at Loughborough University.
Blood, urine and saliva samples will be divided into aliquots in smaller vials, to be stored at -80ºC, and identified only with a code. Your name and personal details will be in no way associated with the physical samples.

Data from the heart rate monitor and blood pressure cuff will be processed at the Department of Chemistry at Loughborough University, using only your experiment identifier.
Skin conductivity and skin tremor data will be processed at Unilever Research, Port Sunlight, Merseyside. Please note that nothing more than a created participant identification number will be used and Unilever will not receive any personal information of yours.
All samples processed at the Department of Chemistry at Loughborough University will be destroyed in an appropriate manner, which is approved by ethics.

Your name will be removed, and the samples identified only with a code. No genetic testing with 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. This information will be kept in a separate locked drawer.
The measurement data will only be made available to people who are directly involved with the study.

Who is organising and funding the research?
The study is collaboration between the Department of Chemistry and the School of Sport, Exercise and Health Science at Loughborough University.

Who has reviewed the study?
This study has been reviewed and has 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
Centre for Analytical Sciences, Department of Chemistry, Loughborough University
LE11 3TU, Tel: 01509 222 549
Thank you for taking time to read this information sheet and considering helping us with our research. Please retain a copy for your reference.

Chapter 5 Non-invasive fast skin profiling for elements from the volar and plantar and 5.7.Non-invasive fast skin profiling for elements from the axilla, dorsal, plantar and volar

What is the purpose of the study?

The researchers intend to test the effectiveness of a new, non-invasive skin odour sampling device called the Liquid Skin Pen (see Figure 1, overleaf). This is a hand-held device that uses a gentle flow of ethanol and water to sample the range of chemical compounds that are present on, and evaporate from, human skin. The researchers intend to use the Liquid Skin Pen to study the range of chemicals released from healthy human skin.

Normal metabolic processes result in the release, *via* the skin surface, of a wide range of chemical compounds, a large proportion of which are classed as volatile organic compounds (VOCs). These compounds may be derived directly from the diet, or they may be formed during the day-to-day functioning of individual bodily organs. A number of these chemicals contribute to the characteristic odour of the human body.

It is known that this chemical profile is dynamic – an individual's profile (and hence odour) fluctuates according to diet, alteration in skin microbial populations, and time of year. Of greatest interest, though, is the fact that some internal metabolic conditions also induce changes in the nature of this chemical profile. Once we understand the nature of a healthy, normal human skin chemical profile, we can begin to look for chemical indicators of metabolic change.

For this study, the researchers are looking at how effectively the Liquid Skin Pen samples the odour of healthy human skin.

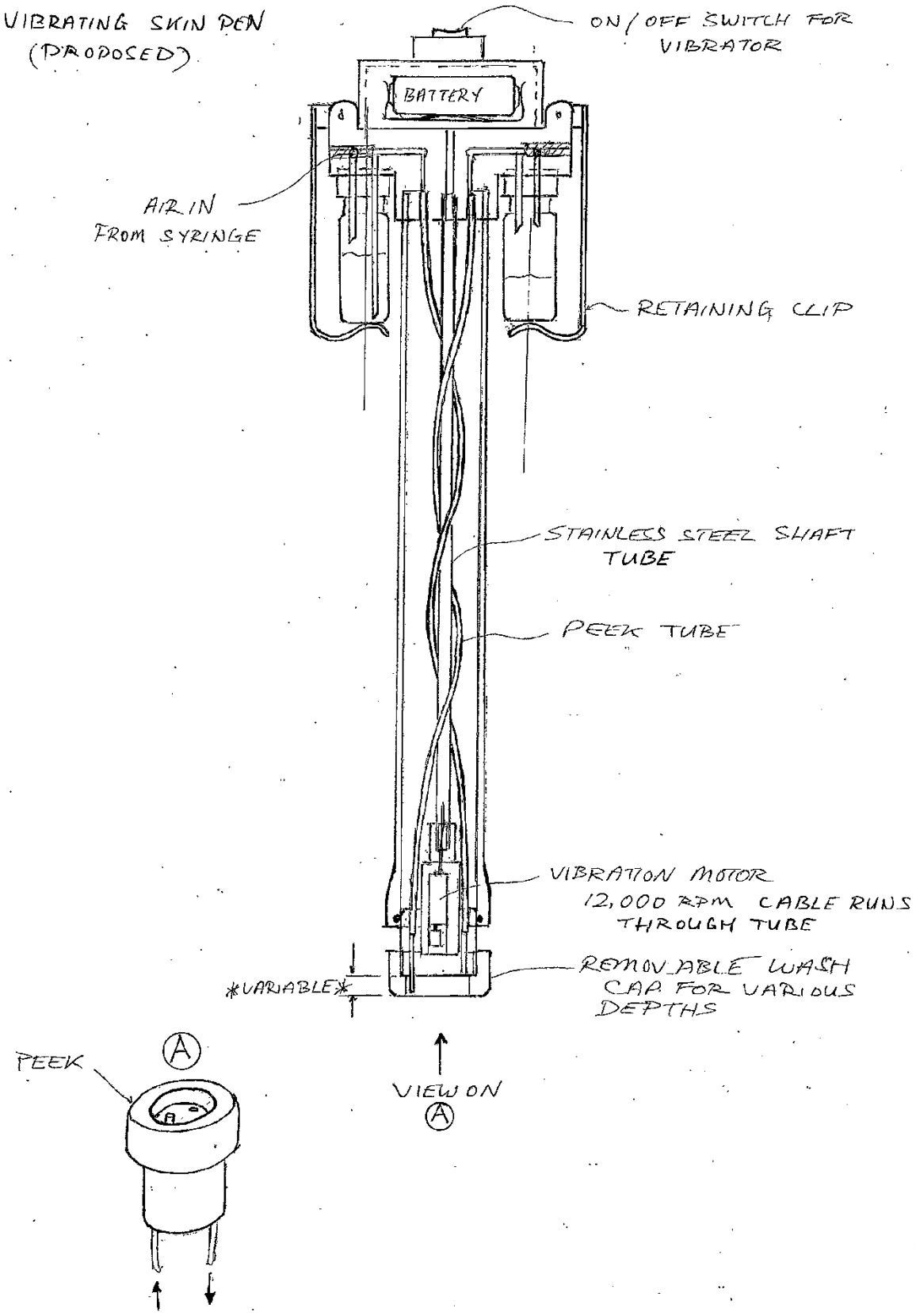


Figure 8.3. Schematic of the Liquid Skin Pen

Who is doing this research and why?

This study is sponsored by Unilever. The project leader is Professor Paul Thomas, of Loughborough University's Centre for Analytical Science. The researchers intend to test the effectiveness of a new, non-invasive skin odour sampling device called the Liquid Skin Pen.

Are there any exclusion criteria?

We require healthy male participants between the ages of 18 and 65. Participants with sensitive skin will be excluded.

Once I take part, can I change my mind?

Yes, of course. After you have read this information, and asked any questions you may have, we will ask you to complete an Informed Consent Form. However, if at any time before, during or after the sessions you wish to withdraw from the study, please contact the main investigator, Professor Thomas. You can withdraw at any time, for any reason, and you will not be asked to explain your reasons for withdrawing.

Will I be required to attend any sessions and where will these be?

You will be asked to come to Room F2.08 in the Department of Chemistry at Loughborough University.

How long will it take?

The entire procedure should be completed within 2 hours.

Is there anything I need to do before the sessions?

We would require you to fast from midnight on the night before the session. Water, however, will be allowed.

What will I be asked to do?

At the familiarisation session

You will come to Room F2.08, and we will show you around the laboratory. You will have the opportunity to see the Skin Pen and meet the researchers involved in the study. Please feel free to contact Professor Thomas or Dr Burns if you have any questions after the familiarisation session.

You will be asked to fast overnight. Water, however, will be allowed.

At the testing session
The following morning in Room F2.08, at the beginning of the proceedings, you will be asked to complete a Health Screen Questionnaire and to sign a Consent Form.

We will then wash your skin with distilled water, and dry the skin using cotton wool. The exact region of your skin to be sampled will be discussed and agreed with you beforehand;

typical regions for sampling would include the underarm, forearm and back. You will then be asked to return to the laboratory in two hours, at which point sampling will begin.

Each skin surface will be sampled for 5 minutes. We will map the odour of the body by taking samples from several surfaces. The total sampling time will amount to no more than 75 minutes.

What personal information will be required from me?

We will take your name, age and contact details. Participants will be required to complete a Health Screen Questionnaire.

Are there any risks in participating?

We do not anticipate any risks associated with participating in this study.

Will my taking part in this study be kept confidential?

Absolutely. Although we will record your name, age and contact details, these will be stored in a secure place to which only the researchers involved in the study will have access. You will be assigned a Participant Identifier code, and this code will be used instead of your name to identify the samples during analysis and processing.

What will happen to the results of the study?

The researchers will input the results into a database, which will describe the compounds detected from samples of healthy human skin. At some stage, the researchers may publish the results of the study, but be assured that your name will not be included in any publication.

What do I get for participating?

Your contribution to this research may ultimately help clinicians diagnose, in a non-invasive way, the earliest stages of metabolic change - potentially saving lives in the future.

I have some more questions. Who should I contact?

Please contact Professor Paul Thomas on 01509 222549, (C.L.P.Thomas@lboro.ac.uk), or Dr Corrinne Burns, on 01509 222590 (C.Burns@lboro.ac.uk)

What if I am not happy with how the research was conducted?

The University has a policy relating to Research Misconduct and Whistle Blowing which is available online at http://www.lboro.ac.uk/admin/committees/ethical/Whistleblowing(2).htm. Please ensure that this link is included on the Participant Information Sheet.

Main Investigator:

Professor Paul Thomas, Centre for Analytical Science, Department of Chemistry, Loughborough University, Leicestershire LE11 3TU. Telephone: 01509 222549

Additional Investigators:

Professor Colin Creaser, Centre for Analytical Science, Department of Chemistry, Loughborough University, Leicestershire LE11 3TU

Helen Martin, Doctoral Student, Centre for Analytical Science, Department of Chemistry, Loughborough University, Leicestershire LE11 3TU

Pareen Patel, Doctoral Student, Centre for Analytical Science, Department of Chemistry, Loughborough University, Leicestershire LE11 3TU

Dr Corrinne Burns, Centre for Analytical Science, Department of Chemistry, Loughborough University, Leicestershire LE11 3TU. Telephone: 01509 222590

Chapter 5 Section 5.8 Non-invasive fast profiling of the dorsal and plantar for semi-volatile organic compounds and non-volatile organic compounds

What is the purpose of the study?

The researchers intend to test the effectiveness of a new, non-invasive skin odour sampling device called the Liquid Skin Pen (see Figure 1, overleaf). This is a hand-held device that uses a gentle flow of ethanol and water to sample the range of chemical compounds that are present on, and evaporate from, human skin. The researchers intend to use the Liquid Skin Pen to study the range of chemicals released from healthy human skin.

Normal metabolic processes result in the release, *via* the skin surface, of a wide range of chemical compounds, a large proportion of which are classed as volatile organic compounds (VOCs). These compounds may be derived directly from the diet, or they may be formed during the day-to-day functioning of individual bodily organs. A number of these chemicals contribute to the characteristic odour of the human body.

It is known that this chemical profile is dynamic – an individual's profile (and hence odour) fluctuates according to diet, alteration in skin microbial populations, and time of year. Of greatest interest, though, is the fact that some internal metabolic conditions also induce changes in the nature of this chemical profile. Once we understand the nature of a healthy, normal human skin chemical profile, we can begin to look for chemical indicators of metabolic change.

For this study, the researchers are looking at how effectively the Liquid Skin Pen samples the odour of healthy human skin.

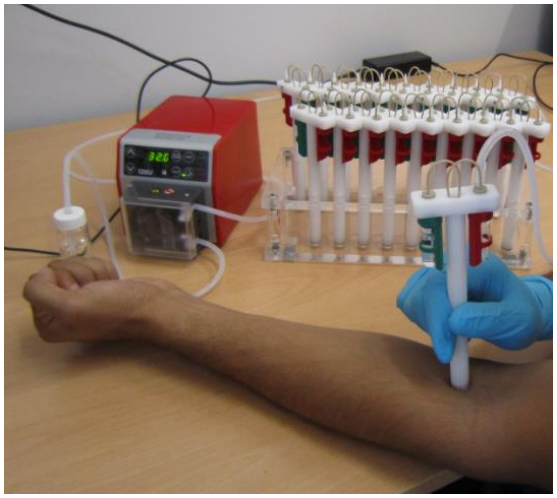


Figure 1. Skin sampling using the liquid skin pen

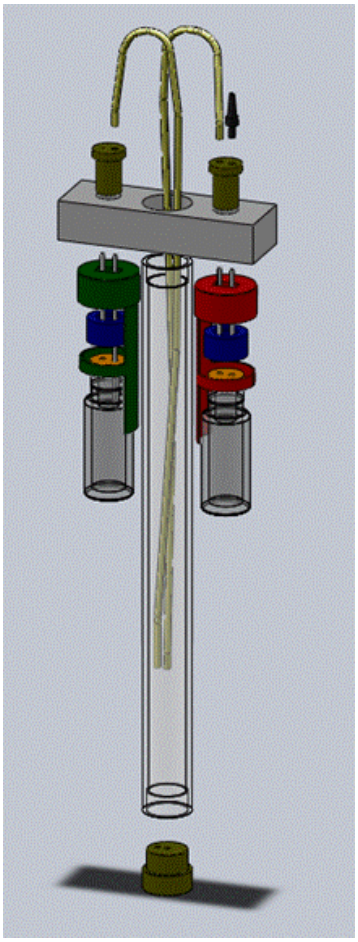
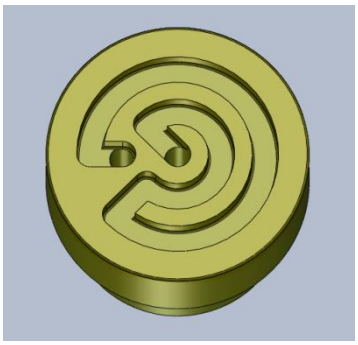


Figure 2. The liquid skin pen (above) and the sampling base (below)



Who is doing this research and why?

This study is sponsored by Unilever. The project leader is Professor Paul Thomas, of Loughborough University's Centre for Analytical Science. The researchers intend to test the effectiveness of a new, non-invasive skin odour sampling device called the Liquid Skin Pen.

Are there any exclusion criteria?

We require healthy male and female participants between the ages of 18 and 65. Participants with sensitive skin will be excluded.

Once I take part, can I change my mind?

Yes, of course. After you have read this information, and asked any questions you may have, we will ask you to complete an Informed Consent Form. However, if at any time before, during or after the sessions you wish to withdraw from the study, please contact the main investigator, Professor Thomas. You can withdraw at any time, for any reason, and you will not be asked to explain your reasons for withdrawing.

Will I be required to attend any sessions and where will these be?

You will be asked to come to Room F2.08 in the Department of Chemistry at Loughborough University for 1 sampling session.

How long will it take?

The entire procedure should be completed within 1 hour.

Is there anything I need to do before the sessions?

You are asked to cover your feet (including during sleeping hours) for two days before the trial. Socks and soap (please do not use any other soap or shampoo during this time) will be provided.

What will I be asked to do?

You will be asked to fast overnight. Water, however, will be allowed.

At the testing session
The following morning in Room F2.08, at the beginning of the proceedings, you will be asked to complete a Health Screen Questionnaire and to sign a Consent Form.

We will then wash your skin with distilled water, and dry the skin using cotton wool. The exact region of your skin to be sampled will be discussed and agreed with you beforehand; typical regions for sampling would include the top and bottom of one foot. Sampling will then commence.

Each skin surface will be sampled for approximately 5 minutes. We will map the odour of the feet by taking samples from the top and the base. The total sampling time will amount to no more than 10 minutes.

What personal information will be required from me?

We will take your name, age and contact details. Participants will be required to complete a Health Screen Questionnaire.

Are there any risks in participating?

We do not anticipate any risks associated with participating in this study.

Will my taking part in this study be kept confidential?

Absolutely. Although we will record your name, age and contact details, these will be stored in a secure place to which only the researchers involved in the study will have access. You will be assigned a Participant Identifier code, and this code will be used instead of your name to identify the samples during analysis and processing.

What will happen to the results of the study?

The researchers will input the results into a database, which will describe the compounds detected from samples of healthy human skin. At some stage, the researchers may publish the results of the study, but be assured that your name will not be included in any publication.

What do I get for participating?

Your contribution to this research may ultimately help clinicians diagnose, in a non-invasive way, the earliest stages of metabolic change - potentially saving lives in the future.

I have some more questions. Who should I contact?

Please contact Professor Paul Thomas on 01509 222549, (C.L.P.Thomas@lboro.ac.uk), or Pareen Patel, on 01509 222556 (P.Patel@lboro.ac.uk)

What if I am not happy with how the research was conducted?

The University has a policy relating to Research Misconduct and Whistle Blowing which is available online at http://www.lboro.ac.uk/admin/committees/ethical/Whistleblowing(2).htm.

Main Investigator:

Professor Paul Thomas, Centre for Analytical Science, Department of Chemistry, Loughborough University, Leicestershire LE11 3TU. Telephone: 01509 222549

Additional Investigators:

Professor Colin Creaser, Centre for Analytical Science, Department of Chemistry, Loughborough University, Leicestershire LE11 3TU

Dr James Reynolds, Centre for Analytical Science, Department of Chemistry, Loughborough University, Leicestershire LE11 3TU

Pareen Patel, Doctoral Student, Centre for Analytical Science, Department of Chemistry, Loughborough University, Leicestershire LE11 3TU

PASAT Instruction

The participants are given these standardised instructions:

On the tape you will hear a series of numbers, you must add the most recent numbers together and say the answers out loud, for example if you hear 4.....6..... you say 10. You then must remember the second number, in this case 6, and add it to the next number you hear.

It is important that you speak clearly and answer a quickly as possible, this is a performance test and you will be marked against other participants.

There will be a live video feed of your face played on the screen in front of you, you will be asked to look at this at all times during the PASAT.

We will take your blood pressure 4 times during the test but will be unable to warn you as it will interfere with the test so do not be alarmed if you feel the blood pressure cuff tightening. If you feel more uncomfortable than during the previous blood pressure measurements please let us know.

The test will begin with a 30 second practice clip and then we will move onto the live test.

Do you have any questions before we begin?

The participant is given the practice test then given another chance to ask questions before the live test begins.

During the live test wrong answers are indicated by a loud blast on a horn, this often overlaps with the next number causing more errors – this is designed to frustrate and disorientate the participant. On the answer sheet you will observe series of boxes highlighted in grey, during these times the horn will be sounded for a correct answer as well.

The boxes shaded in black on the answer sheet indicate the point at which blood pressure readings are started.

During the study visit the participant completes a set of cognitive function tests, then the PASAT (or placebo) then the same cognitive function tests again. The sensewear™ was applied to the underside of the upper arm when the participants arrived. The time stamp was pressed at the start of each cognitive battery and at the start of the PASAT (or placebo). Attached is a log of the sensewear™ usage. It is important to note that many participants found the final cognitive function test (~9min after the start of the cognitive battery) difficult and many said they found it stressful.

The placebo condition was designed to last the same time as the PASAT practise and live test combined. It is a continuous track of music designed to be relaxing, participants were only monitored by their blood pressure at the same time intervals as for the PASAT, other than that they were encouraged to sit comfortably and relax, windows media player visual effects were on the screen in front of them but they were not encouraged or discouraged from watching them.

Participant Code: _____ Date: _____

4	Answer		6	8		9	18		2	3	
6	10		6	12		3	12		9	11	
3	9		2	8		8	11		2	11	
1	4		4	6		1	9		9	11	
7	8		6	10		3	4		1	10	
3	10		3	9		2	5		2	3	
3	6		3	6		5	7		4	6	
2	5		5	8		3	8		5	9	
2	4		1	6		5	8		4	9	
4	6		5	6		7	12		7	11	
4	8		8	13		4	11		9	16	
9	13		5	13		6	10		8	17	
5	14		9	14		6	12		3	11	
7	12		4	13		4	10		6	9	
1	8		8	12		8	12		2	8	
8	9		9	17		5	13		1	3	
8	16		7	16		7	12		6	7	
6	14		7	14		8	15		1	7	
9	15		3	10		3	11		3	4	
5	14		4	7		9	12		6	9	
5	10		8	12		6	15		7	13	
4	9		3	11		6	12		4	11	
5	9		1	4		1	7		2	6	
6	11		8	9		4	5		9	11	
7	13		6	14		3	7		8	17	
3	10		7	13		7	10		4	12	
3	6		8	15		9	16		7	11	
6	9		8	16		9	18		2	9	
8	14		3	11		3	12		3	5	
9	17		7	10		5	8		7	10	
1	10		7	14		2	7		3	10	
5	6		9	16		2	4		7	10	
3	8		5	14		3	5		8	15	
7	10		4	9		7	10		8	16	
2	9		1	5		4	11		2	10	
9	11		1	2		8	12		2	4	
2	11		6	7		1	9		3	5	
8	10		2	8		8	9		6	9	
1	9		4	6		8	16		5	11	
9	10		2	6		5	13		4	9	
8	17		5	7		6	11		1	5	
7	15		4	9		7	13		2	3	
1	8		3	7		2	9		5	7	
6	7		6	9		4	6		5	10	
4	10		7	13		3	7		2	7	
2	6		8	15		7	10		9	11	
4	6		2	10		3	10		7	16	
1	5		3	5		7	10		7	14	
7	8		6	9		8	15		2	9	
1	8		1	7		3	11		7		
9	10		5	6		4	7				
2	11		8	13		5	9		Total		
2	4		9	17		1	6		10min		

Figure 8.4. PASAT answer sheet

Health Screen Questionnaire for study volunteers

Participant identifier: .....

Trial Supervisor

Prof. Paul Thomas, Professor Analytical Science, Department of Chemistry, Loughborough University, LE11 3TU

Tel. 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 current mouth sores, dental problems, gingivitis or gum disease?	Yes	No
Do you have any specific dietary preferences? (e.g., vegan, vegetarian)	Yes	No
Do you have any chest or breathing problems?	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 (except contraception)	Yes	No

Have you ever suffered from any of the following:

Heart or blood pressure problems?	Yes	No
Convulsions or epilepsy?	Yes	No
Asthma?	Yes	No
Eczema?	Yes	No
Disturbance of vision?	Yes	No
Ear or hearing problems?	Yes	No
Diabetes?	Yes	No
Blood disorders?	Yes	No
Head injury?	Yes	No
Kidney or liver problems?	Yes	No

If you answered 'Yes' to any of the above could you please provide further information :

.....

Do you have any allergies that you are aware of? Yes No

If YES please provide details here: (include allergies to food, medicines and plasters)

.....

Is there anything else relating to your health that you feel we should be aware of?

.....

Has any otherwise healthy member of your family under the age of 25 died suddenly during or soon after exercise? Yes No

If YES please describe the circumstances briefly if you feel able

.....

Please provide contact details of a suitable person for us to contact in the event of any incident or emergency.

Name:.....

.....

Telephone

Number:.....

Relationship to

Participant.....

Are you currently involved in any other studies at the University? Yes No

If yes please provide details of the study

.....

.....

Please inform us of any changes in the above before the study starts.

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 VI Patent

Schedule 1

Details of Invention

Intellectual property as identified within the patent application generated from the research undertaken under the Sponsored Research Agreement - University Ref: J12552; Unilever UK Central Resources Ltd Ref: PS-2010-0126 for the project entitled "Skin Pen Development" during the period 2010 – 2011.
(Please note, the results of this collaboration belong to Unilever.)

Title and Abstract of Patent: Skin Surface Sampling System

"The present invention provides a skin surface sampling device in which a supply tube and a recovery tube are housed in an elongated hand-holdable tubular sleeve, the sleeve having upper and lower ends positioned along a longitudinal axis and the lower end being provided with a sampling head;
in which:
the supply tube is adapted to convey liquid from a liquid source to the sampling head;
the recovery tube is adapted to convey liquid from the sampling head to a liquid collection vessel;
the sampling head has a planar surface adapted for application to the surface of the skin to form a contact
therewith during sampling;
the planar surface has a liquid entry port adapted for liquid communication with the supply tube and a liquid
exit port adapted for liquid communication with the recovery tube;
and characterised in that:
the liquid entry port and the liquid exit port are connected via a guide channel which is adapted to
guide liquid from the liquid entry port, along the surface of the skin for the elution of materials on the skin
surface, and then to the liquid exit port for recovery.

The device can be used in a rapid and non-invasive system of skin surface sampling, enabling the sampling of a wide range of compounds (including but not limited to VOCs and SVOCs) from the surface of the skin, and which can be used over all areas of the body."

Inventors:	Corrinne Burns	(University)
	Peter David Carroll	(Company)
	Pareenkumar Patel	(University)
	Svetlana Riazanskaia	(Company)
	Charles Laurence Paul Thomas	(University)

Applicant: Unilever Plc
Unilever House, 100 Victoria Embankment, London, EC4Y 0DY, UK

Filing Date: 10 May 2012

Application Number: EP12167428

Unilever Plc Case No.: T3269(C)

LU Technology ID No: 714

SCHEDULE

Intellectual property as identified within the patent application generated from the research undertaken under the Sponsored Research Agreement - University Ref: J12552 (Tech ID No. 714); Company Ref: PS-2010-0126 for the project entitled “Skin Pen Development” during the period 2010 – 2011.

(Please note, the results of this collaboration belong to Unilever.)

PATENT APPLICATION:

Title of Invention:	Skin Surface Sampling System	
Application Number:	EP12167428	
Filing Date:	10 May 2012	
Inventors:	Corrinne Burns	(University)
	Peter David Carroll	(Company)
	Pareenkumar Patel	(University)
	Svetlana Riazanskaia	(Company)
	Charles Laurence Paul Thomas	(University)
Applicant:	Unilever Plc Unilever House, 100 Victoria Embankment, London, EC4Y 0DY, UK	
Case No.:	T3269(C)	

EP Abstract

“The present invention provides a skin surface sampling device in which a supply tube and a recovery tube are housed in an elongated hand-holdable tubular sleeve, the sleeve having upper and lower ends positioned along a longitudinal axis and the lower end being provided with a sampling head;
in which:
the supply tube is adapted to convey liquid from a liquid source to the sampling head;
the recovery tube is adapted to convey liquid from the sampling head to a liquid collection vessel;
the sampling head has a planar surface adapted for application to the surface of the skin to form a contact
therewith during sampling;
the planar surface has a liquid entry port adapted for liquid communication with the supply tube and a liquid
exit port adapted for liquid communication with the recovery tube;
and characterised in that:
the liquid entry port and the liquid exit port are connected via a guide channel which is adapted to
guide liquid from the liquid entry port, along the surface of the skin for the elution of materials on the skin
surface, and then to the liquid exit port for recovery.

The device can be used in a rapid and non-invasive system of skin surface sampling, enabling the sampling of a wide range of compounds (including but not limited to VOCs and SVOCs) from the surface of the skin, and which can be used over all areas of the body.”

Appendix VII Publications

Published Papers

1 A. Malkar, N. A. Devenport, H. J. Martin, P. Patel, M. A. Turner, P. Watson, R. J. Maughan, H. J. Reid, B. L. Sharp, C. L. P. Thomas, J. C. Reynolds, and C. S. Creaser, Metabolomics, 2013.

2 M. A Turner, S. Bandelow, L. Edwards, P. Patel, H. J. Martin, I. D. Wilson, and C. L. P. Thomas, J. Breath Res., 2013, 7, 017102.

3 R. Huo, A. Agapiou, V. Bocos-Bintintan, L. J. Brown, C. Burns, C. S. Creaser, N. A Devenport, B. Gao-Lau, C. Guallar-Hoyas, L. Hildebrand, A. Malkar, H. J. Martin, V. H. Moll, P. Patel, A. Ratiu, J. C. Reynolds, S. Sielemann, R. Slodzynski, M. Statheropoulos, M. A Turner, W. Vautz, V. E. Wright, and C. L. P. Thomas, J. Breath Res., 2011, 5, 046006.

Publications in preparation

1. P. Patel, C. Burns, S. Riazanskaia, P. Carroll, J. C. Reynolds, H. J. Reid and C. L. P. Thomas, Non-invasive fast site specific skin profiling for semi-volatile organic compounds, elements and non-volatile organic compounds.

2. P. Patel, A. Malkar, H. J. Martin, M. A. Turner, P. Watson, R. J. Maughan, C. S. Creaser, B. L. Sharp, H. J. Reid and C. L. P. Thomas, Elemental profiling of human skin, saliva, plasma and urine before and after induced physiological stress by inductively coupled plasma mass spectrometry

3. P. Patel, A. Malkar, H. J. Martin, M.A. Turner, P. Watson, R. J. Maughan, C. S. Creaser, B. L. Sharp, H.J. Reid and C. L. P. Thomas, Analysis of human saliva profiles before and after induced physiological stress by inductively coupled plasma mass spectrometry, thermal desorption-gas chromatography-mass spectrometry and ultra-high performance liquid chromatography-ion mobility-mass spectrometry

Discovery of Stress Biomarkers in Biological Matrices using Novel Sample Collection Techniques, Inorganic and Organic Mass Spectrometry and Multivariate Analysis

Pareen Patel

A Doctoral Thesis

Supplementary Information

Submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of Loughborough University

November 2013

Table of Contents

List of Tables

Table of Figures

1 Statistical Analysis

Analytical methods should be free from systematic error meaning the value given for the amount of analyte should be the true value. However, even if no systematic errors are present, random errors make it unlikely that the achieved value would exactly equal to a reference amount (a standard or experimental value). In order to determine whether these differences can be attributed to random variations, a significant test can be employed.

Significance tests are testing the truth of a hypothesis which is known as the null hypothesis (H_0). The term null implied there is no difference between the observed and known (or second experimental) values other than that which can be attributed to random variation. Assuming H_0 is true, statistical theory can be utilized to calculate the probability that the differences between the observed and known values are due to random errors. The lower the probability that the differences occur by chance the less likely H_0 is true.

1.1.1.1 Comparison of experimental means (t-test)

One method to employ significance tests is to compare an obtained value with a second value obtained from a different method (i.e. second sample site) with the test containing two experimental means $\bar{x}_1$ and $\bar{x}_2$. To test whether the two methods give the same result, i.e.H_0: $\mu_1 = \mu_2$, a test whether ($\bar{x}_1$ - $\bar{x}_2$) differs significantly from zero, the statistical t is calculated. If the two samples have standard deviations which are not significantly different, a pooled estimate (s) of the standard deviation can be calculated from the two individual standard deviations s_1 and s_2.

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \qquad \text{(Equation SI.2.1)}$$

where s is calculated from

$$s^2 = \frac{(n_1 - 1){s_1}^2 + (n_2 - 1){s_2}^2}{(n_1 + n_2 - 2)}$$

Where n = number of samples and t has $n_1 + n_2 - 2$ degrees of freedom. This method assumes that the samples are drawn from populations with equal standard deviations. If standard deviations are likely to be equal than it is no longer suitable to use a pooled standard deviation and a different method giving an overall estimate of the standard deviation should be employed (Equation SI.2.2).

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{s\sqrt{\frac{{s_1}^2}{n_1} + \frac{{s_2}^2}{n_2}}} \qquad \text{(Equation SI.2.2)}$$

With degrees of freedom (df)

$$\mathrm{df} = \frac{\left(\frac{{s_1}^2}{n_1} + \frac{{s_2}^2}{n_2}\right)^2}{\left(\frac{{s_1}^4}{{n_1}^2(n_1 - 1)} + \frac{{s_2}^2}{{n_2}^2(n_2 - 1)}\right)}$$

With the value obtained being truncated to an integer.

1.1.1.2 Comparison of standard deviation (F-test)

As well as comparing the systematic errors of two means it is also important to compare the random errors by comparing the standard deviations of two sets of data i.e. to test whether Method A and Method B differ in their precision. To test whether the difference between two sample variances is significant, i.e. to test $H_0: {\sigma_1}^2 = {\sigma_2}^2$, the statistic F is calculated:

$$F = \frac{{s_1}^2}{{s_2}^2} \qquad \text{(Equation SI.2.3)}$$

where 1 and 2 are allocated in the equation so that F is always ≥ 1. Degrees of free of the numerator and denominator are $n_1 - 1$ and $n_2 - 1$, respectively.

1.1.1.3 Analysis of Variance

Often more than two means need to be compared e.g. comparing the mean results obtained of analyte concentration from several different target sites, where there are two possible sources of variation. The first is random error and the second possible source of variation is due to controlled (fixed-effect) factor such as the target site used. Analysis of variation (ANOVA) can be used to separate and estimate the different causes of variation. It can be used to separate any variation which is caused by changing the controlled factor from the variation due to random error thus used to test whether significant differences between mean values obtained result from altering the controlled factor.

When two factors can affect the experimental result, their effect must be studied by employing two way ANOVA (Table SI.1.1). Each of the *N* measurements, X_{ij}, is classified under treatment levels (c) and blocks (r), so N = cr. The row totals ($T_{1.}$, $T_{2.}$, etc.) and column totals ($T_{.1}$, $T_{.2}$, etc.), and the grand total, T, are given and used in the calculation.

Table SI.1.1. Formulae for two-way ANOVA

Source of variation	Sum of squares	Degrees of freedom
Between-treatment	$\Sigma T_{.j}^{\,2}/r - T^2/N$	c – 1
Between-block	$\Sigma T_{i.}^{\,2}/c - T^2/N$	r – 1
Residual	By subtraction	by subtraction
Total	$\Sigma\Sigma x_{ij}^{\,2} - T^2/N$	*N* -1

where N = nh = total number of measurements

T_i = sum of all measurements in the *i*th sample

T = sum of all the measurements, grand total.

The test statistic is

$$F = \frac{between - sample\ mean\ square}{within - sample\ mean\ square} \qquad \text{(Eq. 2.4)}$$

And the critical value is $F_{h-1,N-h}$.

2 A study into the effect of psychological stress on the elemental content of human forehead sweat as measured by ICP-MS

Table SI.2.1. Participant information summary for the elemental profiling of sweat, by ICP-MS, obtained from psychological stress

Participant	Visit 1					Visit 2					Gender	No. of samples	Sample Storage
	Sample Date	Sample ID	Sample Day	Sample Time	Intervention	Sample Date	ID	Sample Day	Sample Time	Intervention			
1	10/02/2010	TE03 P	1	0900-1100	PASAT	23/03/2010	TE03 O	29	0900-1100	Placebo	Male	4	2% HNO_3 at RT
2	11/02/2010	TE04 O	2	0900-1100	Placebo	25/03/2010	TE04 P	31	0900-1100	PASAT	Female	4	2% HNO_3 at RT
3	12/02/2010	TE05 P	3	0900-1100	PASAT	22/03/2010	TE05 O	28	0900-1100	Placebo	Male	4	2% HNO_3 at RT
4	15/02/2010	TE06 O	4	0900-1100	Placebo	23/03/2010	TE06 P	29	1100-1300	PASAT	Male	4	2% HNO_3 at RT
5	15/02/2010	TE07 P	4	1100-1300	PASAT	08/03/2010	TE07 O	17	1100-1300	Placebo	Male	4	2% HNO_3 at RT
6	16/02/2010	TE08 O	5	0900-1100	Placebo	02/03/2010	TE08 P	14	0900-1100	PASAT	Female	4	2% HNO_3 at RT
7	17/02/2010	TE09 P	6	0900-1100	PASAT	24/02/2010	TE09 O	11	0900-1100	Placebo	Female	4	2% HNO_3 at RT
8	16/02/2010	TE10 O	5	1100-1300	Placebo	23/02/2010	TE10 P	10	1100-1300	PASAT	Male	4	2% HNO_3 at RT
9	18/02/2010	TE11 P	7	0900-1100	PASAT	25/02/2010	TE11 O	12	1100-1300	Placebo	Female	4	2% HNO_3 at RT
23	19/02/2010	TE12 O	8	0900-1100	Placebo	No sample obtained as participant did not take part					Male	2	2% HNO_3 at RT
10	22/02/2010	TE13 P	9	1100-1300	PASAT	01/03/2010	TE13 O	13	0900-1100	Placebo	Male	4	2% HNO_3 at RT
11	22/02/2010	TE14 O	9	0900-1100	Placebo	09/03/2010	TE14 P	19	1100-1300	PASAT	Male	4	2% HNO_3 at RT
12	23/02/2010	TE15 P	10	0900-1100	PASAT	02/03/2010	TE15 O	14	1100-1300	Placebo	Female	4	2% HNO_3 at RT
13	25/02/2010	TE16 O	12	0900-1100	Placebo	12/03/2010	TE16 P	22	0900-1100	PASAT	Female	4	2% HNO_3 at RT
14	03/03/2010	TE17 P	15	0900-1100	PASAT	10/03/2010	TE17 O	20	0900-1100	Placebo	Male	4	2% HNO_3 at RT
15	03/03/2010	TE18 O	15	1100-1300	Placebo	10/03/2010	TE18 P	20	1100-1300	PASAT	Female	4	2% HNO_3 at RT
16	05/03/2010	TE19 P	16	0900-1100	PASAT	16/03/2010	TE19 O	24	1100-1300	Placebo	Male	4	2% HNO_3 at RT
17	09/03/2010	TE20 O	18	0900-1100	Placebo	16/03/2010	TE20 P	24	0900-1100	PASAT	Female	4	2% HNO_3 at RT
18	24/03/2010	TE21 P	30	0900-1100	PASAT	26/03/2010	TE21 O	32	0900-1100	Placebo	Female	4	2% HNO_3 at RT
19	11/03/2010	TE22 O	21	0900-1100	Placebo	18/03/2010	TE22 P	26	0900-1100	PASAT	Female	4	2% HNO_3 at RT
20	12/03/2010	TE23 P	22	1100-1300	PASAT	24/03/2010	TE23 O	30	1100-1300	Placebo	Female	4	2% HNO_3 at RT
21	15/03/2010	TE24 O	23	0900-1100	Placebo	17/03/2010	TE24 P	25	1100-1300	PASAT	Female	4	2% HNO_3 at RT
24	15/03/2010	TE25 P	23	1100-1300	PASAT	No sample obtained as participant did not take part					Male	2	2% HNO_3 at RT
22	19/03/2010	TE26 O	27	1100-1300	Placebo	22/03/2010	TE26 P	28	1100-1300	PASAT	Male	4	2% HNO_3 at RT

Table SI.2.2. Skin sample information summary for the elemental profiling of sweat, by ICP-MS, obtained from psychological stress

Sample ID	Sample Date	Time to collect sample (min)	Analysis Date	Analysis order	No. of days in storage
Patch Blank 1	15/06/2010	30	15/06/2010	1	0
Patch Blank 2	15/06/2010	30	15/06/2010	2	0
Patch Blank 3	15/06/2010	30	15/06/2010	3	0
E3 O1	23/03/2010	30	15/06/2010	4	84
E3 O2	23/03/2010	30	15/06/2010	5	84
E3 P1	10/02/2010	30	15/06/2010	6	125
E3 P2	10/02/2010	30	15/06/2010	7	125
E4 O1	11/02/2010	30	15/06/2010	8	124
E4 O2	11/02/2010	30	15/06/2010	9	124
E4 P1	25/03/2010	30	15/06/2010	10	82
E4 P2	25/03/2010	30	15/06/2010	11	82
E5 O1	22/03/2010	30	15/06/2010	12	85
E5 O2	22/03/2010	30	15/06/2010	13	85
E5 P1	12/02/2010	30	15/06/2010	14	123
E5 P2	12/02/2010	30	15/06/2010	15	123
E6 O1	15/02/2010	30	15/06/2010	16	120
E6 O2	15/02/2010	30	15/06/2010	17	120
E6 P1	23/03/2010	30	15/06/2010	18	84
E6 P2	23/03/2010	30	15/06/2010	19	84
E7 O1	08/03/2010	30	15/06/2010	20	99
E7 O2	08/03/2010	30	15/06/2010	21	99
E7 P1	15/02/2010	30	15/06/2010	22	120
E7 P2	15/02/2010	30	15/06/2010	23	120
E8 O1	16/02/2010	30	15/06/2010	24	119
E8 O2	16/02/2010	30	15/06/2010	25	119
E8 P1	02/03/2010	30	15/06/2010	26	105
E8 P2	02/03/2010	30	15/06/2010	27	105
E9 O1	24/02/2010	30	15/06/2010	28	111
E9 O2	24/02/2010	30	15/06/2010	29	111
E9 P1	17/02/2010	30	15/06/2010	30	118
E9 P2	17/02/2010	30	15/06/2010	31	118
E10 O1	16/02/2010	30	15/06/2010	32	119
E10 O2	16/02/2010	30	15/06/2010	33	119
E10 P1	23/02/2010	30	15/06/2010	34	112
E10 P2	23/02/2010	30	15/06/2010	35	112
Patch Blank 1	15/06/2010	30	15/06/2010	36	0
Patch Blank 2	15/06/2010	30	15/06/2010	37	0
Patch Blank 3	15/06/2010	30	15/06/2010	38	0
E11 O1	25/02/2010	30	15/06/2010	39	110
E11 O2	25/02/2010	30	15/06/2010	40	110
E11 P1	18/02/2010	30	15/06/2010	41	117
E11 P2	18/02/2010	30	15/06/2010	42	117
E12 O1	19/02/2010	30	15/06/2010	43	116
E12 O2	19/02/2010	30	15/06/2010	44	116
E13 O1	01/03/2010	30	15/06/2010	45	106
E13 O2	01/03/2010	30	15/06/2010	46	106
E13 P1	22/02/2010	30	15/06/2010	47	113

E13 P2	22/02/2010	30	15/06/2010	48	113
E14 O1	22/02/2010	30	15/06/2010	49	113
E14 O2	22/02/2010	30	15/06/2010	50	113
E14 P1	09/03/2010	30	15/06/2010	51	98
E14 P2	09/03/2010	30	15/06/2010	52	98
E15 O1	02/03/2010	30	15/06/2010	53	105
E15 O2	02/03/2010	30	15/06/2010	54	105
E15 P1	23/02/2010	30	15/06/2010	55	112
E15 P2	23/02/2010	30	15/06/2010	56	112
E16 O1	25/02/2010	30	15/06/2010	57	110
E16 O2	25/02/2010	30	15/06/2010	58	110
E16 P1	12/03/2010	30	15/06/2010	59	95
E16 P2	12/03/2010	30	15/06/2010	60	95
E17 O1	10/03/2010	30	15/06/2010	61	97
E17 O2	10/03/2010	30	15/06/2010	62	97
E17 P1	03/03/2010	30	15/06/2010	63	104
E17 P2	03/03/2010	30	15/06/2010	64	104
E18 O1	03/03/2010	30	15/06/2010	65	104
E18 O2	03/03/2010	30	15/06/2010	66	104
E18 P1	10/03/2010	30	15/06/2010	67	97
E18 P2	10/03/2010	30	15/06/2010	68	97
E19 O1	16/03/2010	30	15/06/2010	69	91
E19 O2	16/03/2010	30	15/06/2010	70	91
E19 P1	05/03/2010	30	15/06/2010	71	102
E19 P2	05/03/2010	30	15/06/2010	72	102
E20 O1	09/03/2010	30	15/06/2010	73	98
E20 O2	09/03/2010	30	15/06/2010	74	98
E20 P1	16/03/2010	30	15/06/2010	75	91
E20 P2	16/03/2010	30	15/06/2010	76	91
E21 O1	26/03/2010	30	15/06/2010	77	81
E21 O2	26/03/2010	30	15/06/2010	78	81
E21 P1	24/03/2010	30	15/06/2010	79	83
E21 P2	24/03/2010	30	15/06/2010	80	83
E22 O1	11/03/2010	30	15/06/2010	81	96
E22 O2	11/03/2010	30	15/06/2010	82	96
E22 P1	18/03/2010	30	15/06/2010	83	89
E22 P2	18/03/2010	30	15/06/2010	84	89
E23 O1	24/03/2010	30	15/06/2010	85	83
E23 O2	24/03/2010	30	15/06/2010	86	83
E23 P1	12/03/2010	30	15/06/2010	87	95
E23 P2	12/03/2010	30	15/06/2010	88	95
E24 O1	15/03/2010	30	15/06/2010	89	92
E24 O2	15/03/2010	30	15/06/2010	90	92
E24 P1	17/03/2010	30	15/06/2010	91	90
E24 P2	17/03/2010	30	15/06/2010	92	90
E25 P1	15/03/2010	30	15/06/2010	93	92
E25 P2	15/03/2010	30	15/06/2010	94	92
E26 O1	19/03/2010	30	15/06/2010	95	88
E26 O2	19/03/2010	30	15/06/2010	96	88
E26 P1	22/03/2010	30	15/06/2010	97	85
E26 P2	22/03/2010	30	15/06/2010	98	85

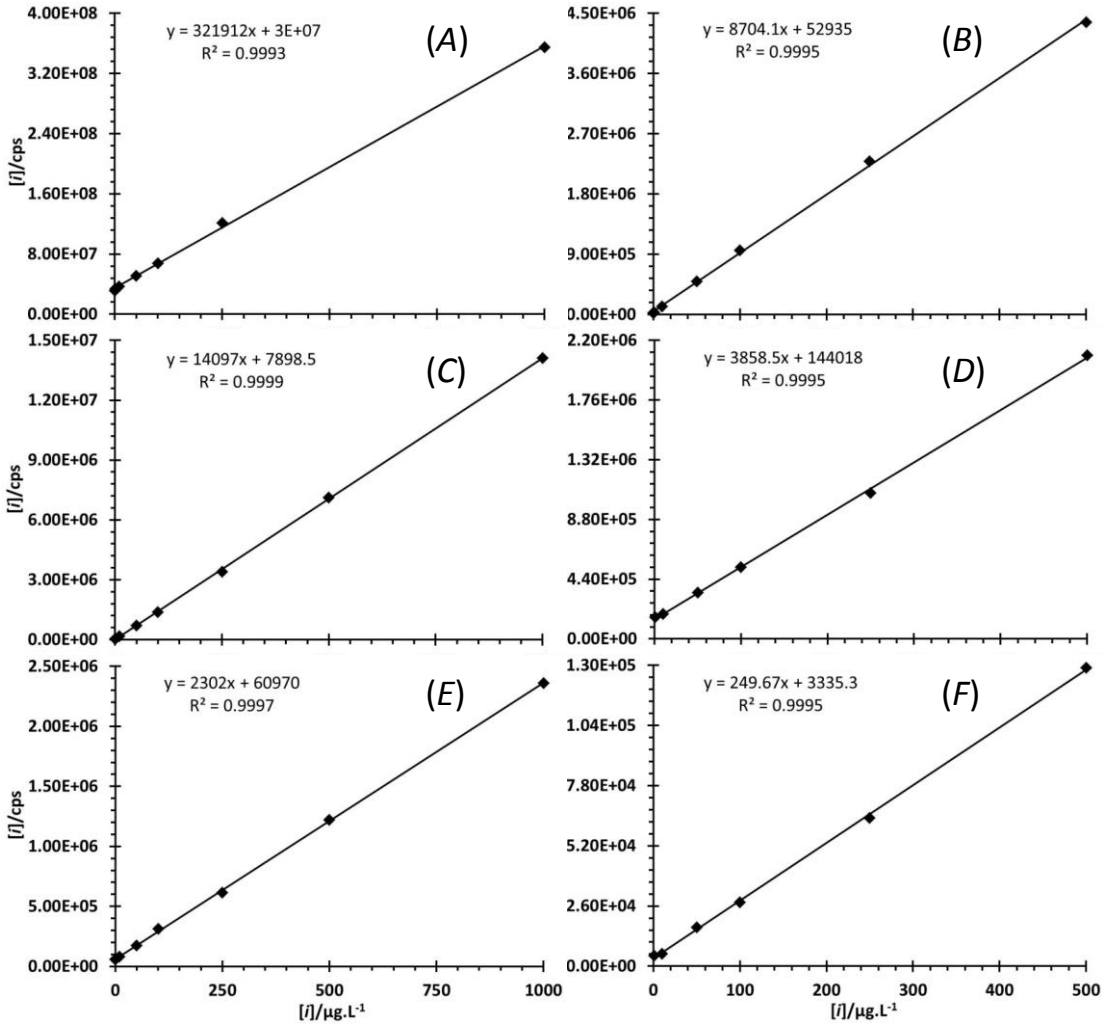


Figure SI.2.1. ICP-MS calibration graphs of (A) sodium (B) zinc (C) potassium (D) sulphur (E) calcium and (F) tin in standards used for calculating elemental concentrations in the skin patches used for psychological stress study

Serially diluted chemical standards (multi-element) were analysed by ICP-MS to establish whether a linear calibration existed for the instrument used. Figure SI.2.1 and Figure SI.2.2 show that for the concentrations of analyte analysed a linear calibration existed for Na, Zn, K, S, Ca, Sn, Ba, Ni, Cu, Ti and Mg. A line of best fit was then be applied to the measured points and the equation of a straight line (y = mx + c) was used to calculate the concentrations of elements from skin patches.

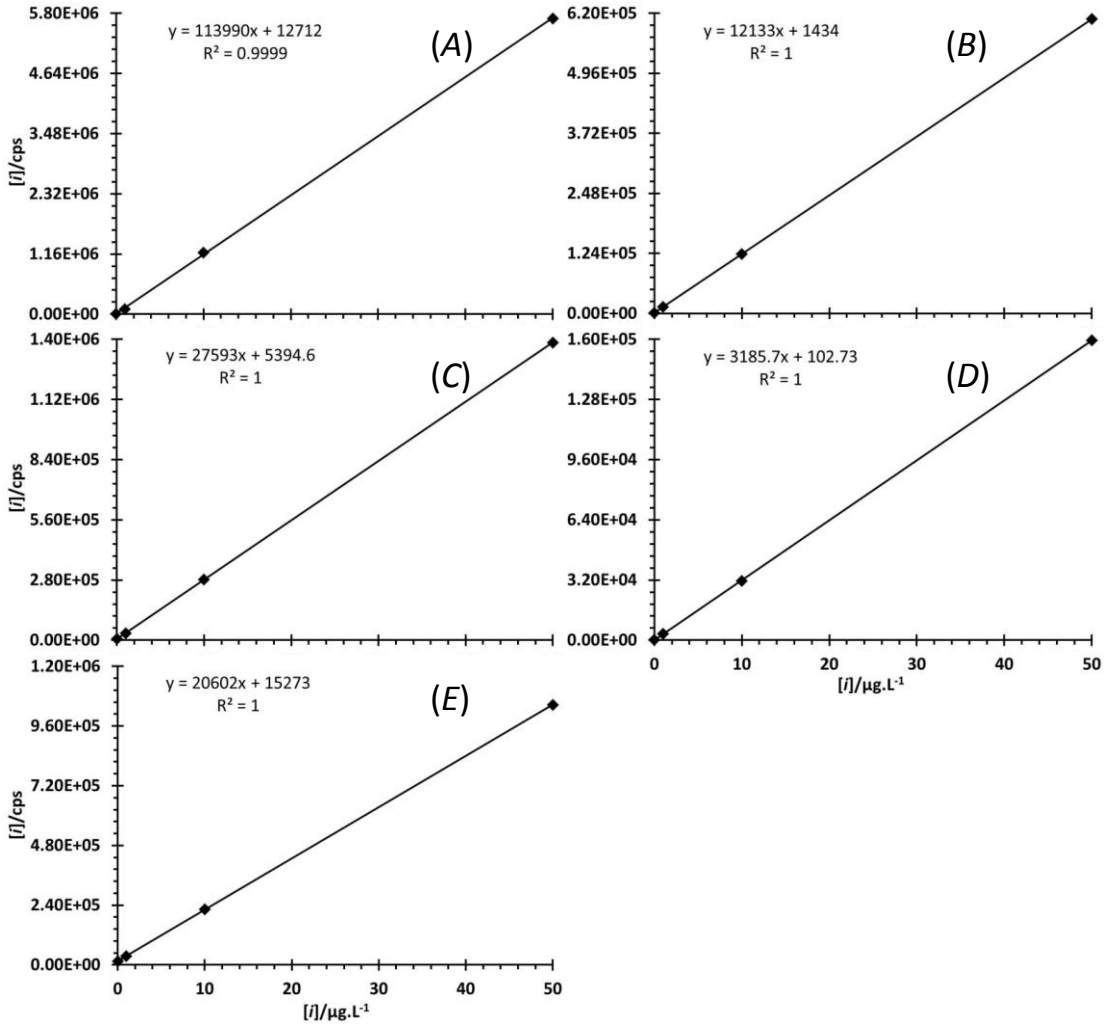


Figure SI.2.2. ICP-MS calibration graphs of (A) barium (B) nickel (C) copper (D) titanium and (E) magnesium in standards used for calculating elemental concentrations in the skin patches used for psychological stress study

3 The effect of physical stress on elemental content in human plasma, skin and urine and the effect on elements, volatile organic compounds and small molecules in human saliva

Table SI.3.1. Sample information summary for the blanking of skin patches

Patch Sample	Sample ID	Sample Date	Sample Day	Sample storage	HNO_3 Mass (g)	Analysis Date	Analysis Order	No. of days in storage
Blank	Patch 0	21/01/2011	1	2% HNO_3	14.0298	09/05/2011	1	108
15% HNO_3	Patch 1	21/01/2011	1	2% HNO_3	14.0303	09/05/2011	2	108
DI H_2O (wash 1)	Patch 2	22/01/2011	2	2% HNO_3	14.0277	09/05/2011	3	107
10% TMAH	Patch 3	23/01/2011	3	2% HNO_3	14.0211	09/05/2011	4	106
DI H_2O (wash 2)	Patch 4	24/01/2011	4	2% HNO_3	14.0066	09/05/2011	5	105
0.1M EDTA (di-Na)	Patch 5	25/01/2011	5	2% HNO_3	14.0375	09/05/2011	6	104
DI H_2O (wash 3)	Patch 6	26/01/2011	6	2% HNO_3	14.0187	09/05/2011	7	103
DI H_2O (wash 4)	Patch 7	27/01/2011	7	2% HNO_3	14.0112	09/05/2011	8	102

Table SI.3.2. Participant information summary for sample collection from physical stress study. M = mass, H = height and R = resistance (R = 2 x M)

Participant	Sample Date	Sample ID	Visit Day	Time	M (kg)	H (m)	R (W)	Age	Total samples & readings	Sample storage
1	31/01/2011	CZ1	1	0715-1015 hrs	69.64	1.77	139	28	66	-80°C
2	31/01/2011	CZ2	1	1030-1330 hrs	73.46	1.74	147	32	67	-80°C
3	01/02/2011	CZ3	2	0715-1015 hrs	82.92	1.89	166	24	64	-80°C
4	01/02/2011	CZ4	2	1030-1330 hrs	83.52	1.96	167	22	67	-80°C
5	02/02/2011	CZ5	3	0715-1015 hrs	72.20	1.74	144	27	68	-80°C
6	02/02/2011	CZ6	3	1030-1330 hrs	74.02	1.82	148	25	67	-80°C
7	03/02/2011	CZ7	4	0715-1015 hrs	92.08	1.92	184	24	66	-80°C
8	03/02/2011	CZ8	4	1030-1330 hrs	88.12	1.78	176	29	60	-80°C
9	04/02/2011	CZ9	5	0715-1015 hrs	100.04	1.77	200	18	65	-80°C
10	04/02/2011	CZ10	5	1030-1330 hrs	88.66	1.68	177	28	60	-80°C
									Total = ***650***	

Table SI.3.3. Skin sample information summary

Sample ID	Sample date	Mass (g) Sample + 2% HNO_3	Internal Standard (In)	Total	Amount In added ($\mu g.L^{-1}$)	Analysis date	Analysis order	No days in storage
CZ1 Blank	31/01/2011	15	0.7017	15.7017	50.2	27/04/2011	84	86
CZ1 Field Blank	31/01/2011	15	0.6998	15.6998	50.1	18/04/2011	29	77
CZ1 A	31/01/2011	15	0.9970	15.9970	70.0	27/04/2011	95	86
CZ1 B	31/01/2011	15	0.7011	15.7011	50.1	20/04/2011	63	79
CZ1 C	31/01/2011	15	0.7027	15.7027	50.3	20/04/2011	64	79
CZ1 D	31/01/2011	15	0.7040	15.7040	50.3	27/04/2011	99	86
CZ2 Blank	31/01/2011	15	0.7019	15.7019	50.2	27/04/2011	77	86
CZ2 Field Blank	31/01/2011	15	0.7029	15.7029	50.3	18/04/2011	17	77
CZ2 A	31/01/2011	15	0.7016	15.7016	50.2	18/04/2011	10	77
CZ2 B	31/01/2011	15	0.7036	15.7036	50.3	20/04/2011	66	79
CZ2 C	31/01/2011	15	0.7028	15.7028	50.3	18/04/2011	31	77
CZ2 D	31/01/2011	15	0.7007	15.7007	50.1	18/04/2011	11	77
CZ3 Blank	01/02/2011	15	0.7015	15.7015	50.2	20/04/2011	68	78
CZ3 Field Blank	01/02/2011	15	0.7032	15.7032	50.3	18/04/2011	32	76
CZ3 A	01/02/2011	15	0.7037	15.7037	50.3	20/04/2011	42	78
CZ3 B	01/02/2011	15	0.7034	15.7034	50.3	20/04/2011	65	78
CZ3 C	01/02/2011	15	0.7028	15.7028	50.3	27/04/2011	85	85
CZ3 D	01/02/2011	15	0.7037	15.7037	50.3	20/04/2011	46	78
CZ4 Blank	01/02/2011	15	0.7046	15.7046	50.4	20/04/2011	59	78
CZ4 Field Blank	01/02/2011	15	0.7045	15.7045	50.4	27/04/2011	102	85
CZ4 A	01/02/2011	15	0.7036	15.7036	50.3	18/04/2011	13	76
CZ4 B	01/02/2011	15	0.7046	15.7046	50.4	18/04/2011	15	76
CZ4 C	01/02/2011	15	0.7025	15.7025	50.2	27/04/2011	100	85
CZ4 D	01/02/2011	15	0.7037	15.7037	50.3	27/04/2011	78	85
CZ5 Blank	02/02/2011	15	0.7031	15.7031	50.3	27/04/2011	101	84
CZ5 Field Blank	02/02/2011	15	0.7035	15.7035	50.3	20/04/2011	43	77
CZ5 A	02/02/2011	15	0.7028	15.7028	50.3	20/04/2011	47	77
CZ5 B	02/02/2011	15	0.7033	15.7033	50.3	27/04/2011	96	84
CZ5 C	02/02/2011	15	0.7039	15.7039	50.3	18/04/2011	14	75

CZ5 D	02/02/2011	15	0.7019	15.7019	50.2	20/04/2011	61	77
CZ6 Blank	02/02/2011	15	0.7041	15.7041	50.4	20/04/2011	60	77
CZ6 Field Blank	02/02/2011	15	0.7037	15.7037	50.3	27/04/2011	80	84
CZ6 A	02/02/2011	15	0.7044	15.7044	50.4	20/04/2011	62	77
CZ6 B	02/02/2011	15	0.7044	15.7044	50.4	20/04/2011	44	77
CZ6 C	02/02/2011	15	0.7027	15.7027	50.3	27/04/2011	83	84
CZ6 D	02/02/2011	15	0.7065	15.7065	50.5	18/04/2011	26	75
CZ7 Blank	03/02/2011	15	0.7051	15.7051	50.4	20/04/2011	50	76
CZ7 Field Blank	03/02/2011	15	0.7038	15.7038	50.3	20/04/2011	45	76
CZ7 A	03/02/2011	15	0.7023	15.7023	50.2	18/04/2011	27	74
CZ7 B	03/02/2011	15	0.7016	15.7016	50.2	20/04/2011	67	76
CZ7 C	03/02/2011	15	0.7042	15.7042	50.4	18/04/2011	8	74
CZ7 D	03/02/2011	15	0.7032	15.7032	50.3	27/04/2011	98	83
CZ8 Blank	03/02/2011	15	0.7030	15.7030	50.3	18/04/2011	25	74
CZ8 Field Blank	03/02/2011	15	0.7037	15.7037	50.3	27/04/2011	93	83
CZ8 A	03/02/2011	15	0.7071	15.7071	50.6	20/04/2011	48	76
CZ8 B	03/02/2011	15	0.7058	15.7058	50.5	27/04/2011	94	83
CZ8 C	03/02/2011	15	0.7034	15.7034	50.3	20/04/2011	49	76
CZ8 D	03/02/2011	15	0.7060	15.7060	50.5	18/04/2011	30	74
CZ9 Blank	04/02/2011	15	0.7055	15.7055	50.5	18/04/2011	34	73
CZ9 Field Blank	04/02/2011	15	0.7024	15.7024	50.2	20/04/2011	51	75
CZ9 A	04/02/2011	15	0.7034	15.7034	50.3	27/04/2011	79	82
CZ9 B	04/02/2011	15	0.7060	15.7060	50.5	27/04/2011	76	82
CZ9 C	04/02/2011	15	0.7034	15.7034	50.3	18/04/2011	28	73
CZ9 D	04/02/2011	15	0.7025	15.7025	50.2	18/04/2011	9	73
CZ10 Blank	04/02/2011	15	0.7063	15.7063	50.5	18/04/2011	12	73
CZ10 Field Blank	04/02/2011	15	0.7058	15.7058	50.5	27/04/2011	97	82
CZ10 A	04/02/2011	15	0.7059	15.7059	50.5	18/04/2011	33	73
CZ10 B	04/02/2011	15	0.7045	15.7045	50.4	27/04/2011	82	82
CZ10 C	04/02/2011	15	0.7055	15.7055	50.5	27/04/2011	81	82
CZ10 D	04/02/2011	15	0.7051	15.7051	50.4	18/04/2011	16	73

Table SI.3.4. Ten blank subtracted elemental skin patch concentrations (µg.L^{-1}) obtained by ICP-MS from ten participants who participated in the physical stress study

Sample ID	Na	Mg	Al	Si	P	S	K	Ca	Cu	Zn
CZ1 A	132	12	0	33	20	52	165	561	0	90
CZ1 B	32921	37	0	17	17	103	3262	327	7	22
CZ1 C	200	0	0	27	0	0	40	0	0	0
CZ1 D	51	0	0	0	0	0	0	0	0	0
CZ2 A	0	0	15	11	0	0	31	91	0	46
CZ2 B	0	79	27	25	30	444	17386	218	4	17
CZ2 C	12636	16	18	0	0	39	1914	124	3	22
CZ2 D	0	0	15	23	0	0	51	76	0	31
CZ3 A	0	0	0	0	0	0	24	0	0	0
CZ3 B	0	128	12	0	29	477	13638	184	3	0
CZ3 C	2271	0	0	0	0	11	241	0	0	0
CZ3 D	0	0	15	63	0	0	0	0	0	0
CZ4 A	0	0	0	0	0	20	19	0	14	872
CZ4 B	0	72	17	12	28	235	11955	343	2	10
CZ4 C	1420	0	0	0	0	9	172	0	0	0
CZ4 D	85	0	0	0	0	12	14	0	0	0
CZ5 A	0	0	0	26	0	15	56	0	0	0
CZ5 B	28855	64	0	0	19	252	8505	412	5	8
CZ5 C	3493	0	0	0	0	32	1115	152	0	30
CZ5 D	0	0	0	0	0	11	40	100	0	11
CZ6 A	0	0	0	0	0	0	0	0	0	0
CZ6 B	14392	42	22	0	11	117	2008	158	4	305
CZ6 C	67	0	0	0	0	0	37	0	0	12
CZ6 D	61	0	0	0	0	0	25	0	0	0
CZ7 A	95	0	0	0	0	0	402	0	0	0
CZ7 B	0	78	0	0	77	313	10332	231	2	0
CZ7 C	4212	0	0	0	15	47	1266	0	0	0
CZ7 D	209	0	0	0	0	0	401	77	0	17
CZ8 A	151	0	0	0	0	11	104	0	0	0
CZ8 B	0	33	0	0	0	480	10215	185	0	0
CZ8 C	14	57	0	17	50	907	5180	714	5	0
CZ8 D	334	0	0	0	0	31	38	0	0	0
CZ9 A	69	0	0	0	0	0	37	0	2	0
CZ9 B	0	87	0	0	42	337	13835	263	4	9
CZ9 C	9397	0	0	0	0	25	1153	21	0	0
CZ9 D	1616	0	0	0	0	0	382	0	0	0
CZ10 A	0	0	0	0	0	0	0	46	0	0
CZ10 B	0	77	0	7	19	272	23655	425	4	0
CZ10 C	0	0	0	32	0	11	4	0	0	0
CZ10 D	0	0	0	0	0	17	0	0	0	0
µ (A)	45	1	1	7	2	10	84	70	2	101
σ (A)	61	4	5	12	6	17	123	175	4	273
µ (B)	7617	70	8	6	27	303	11479	275	4	37
σ (B)	13095	28	11	9	21	136	6347	96	2	94
µ (C)	3371	7	2	8	6	108	1112	101	1	6
σ (C)	4365	18	6	13	16	281	1577	223	2	11
µ (D)	236	0	3	9	0	7	95	25	0	6
σ (D)	497	0	6	20	0	11	157	41	0	11

Table SI.3.5. Saliva sample information summary

Sample ID	Sample date	Mass (g) 2% HNO_3	Saliva	Internal standard (In)	Total	Saliva DF	Amount In added ($\mu g.L^{-1}$)	Analysis date	Analysis Order	No days in storage
CZ1 A R1	31/01/2011	3.6050	0.4093	4.0392	8.0535	19.7	62.0	29/06/2011	10	149
CZ1 A R2	31/01/2011	3.5999	0.3640	4.0038	7.9677	21.9	62.1	07/07/2011	43	157
CZ1 C R1	31/01/2011	3.6048	0.3793	4.0000	7.9841	21.0	61.9	06/07/2011	39	156
CZ1 C R2	31/01/2011	3.6063	0.3008	4.0003	7.9074	26.3	62.5	05/07/2011	30	155
CZ1 D R1	31/01/2011	3.6089	0.4082	4.0041	8.0212	19.7	61.7	28/06/2011	6	148
CZ1 D R2	31/01/2011	3.6001	0.4095	4.0001	8.0097	19.6	61.7	05/07/2011	28	155
CZ2 A R1	31/01/2011	3.6047	0.4015	4.0132	8.0194	20.0	61.8	14/07/2011	50	164
CZ2 A R2	31/01/2011	3.6043	0.3553	4.0073	7.9669	22.4	62.1	07/07/2011	45	157
CZ2 C R1	31/01/2011	3.6033	0.4023	4.0051	8.0107	19.9	61.8	05/07/2011	29	155
CZ2 C R2	31/01/2011	3.6067	0.2861	4.0094	7.9022	27.6	62.7	07/07/2011	46	157
CZ2 D R1	31/01/2011	3.6037	0.4007	4.0104	8.0148	20.0	61.9	29/06/2011	11	149
CZ2 D R2	31/01/2011	3.6067	0.3998	4.0038	8.0103	20.0	61.8	05/07/2011	27	155
CZ3 A R1	01/02/2011	3.6083	0.2150	4.0184	7.8417	36.5	63.3	07/07/2011	48	156
CZ3 D R1	01/02/2011	3.6019	0.3801	4.0028	7.9848	21.0	62.0	30/06/2011	21	149
CZ4 A R1	01/02/2011	3.6016	0.4058	4.0058	8.0132	19.7	61.8	28/06/2011	5	147
CZ4 C R1	01/02/2011	3.6081	0.4082	4.0066	8.0229	19.7	61.7	30/06/2011	24	149
CZ4 C R2	01/02/2011	3.6076	0.4003	4.0058	8.0137	20.0	61.8	05/07/2011	25	154
CZ4 D R1	01/02/2011	3.6042	0.4029	4.0038	8.0109	19.9	61.8	29/06/2011	13	148
CZ4 D R2	01/02/2011	3.6045	0.4020	4.0023	8.0088	19.9	61.7	14/07/2011	51	163
CZ4 D R3	01/02/2011	3.6099	0.4231	4.0002	8.0332	19.0	61.5	07/07/2011	44	156
CZ5 A R1	02/02/2011	3.6014	0.4073	4.0032	8.0119	19.7	61.8	06/07/2011	34	154
CZ5 C R1	02/02/2011	3.6058	0.3717	4.0064	7.9839	21.5	62.0	30/06/2011	22	148
CZ5 D R1	02/02/2011	3.6090	0.2484	4.0413	7.8987	31.8	63.2	30/06/2011	18	148
CZ6 A R1	02/02/2011	3.6092	0.3688	4.0023	7.9803	21.6	62.0	30/06/2011	17	148
CZ6 A R2	02/02/2011	3.6087	0.4066	4.0049	8.0202	19.7	61.7	28/06/2011	2	146
CZ6 C R1	02/02/2011	3.6038	0.3585	4.0073	7.9696	22.2	62.2	28/06/2011	4	146
CZ6 C R2	02/02/2011	3.6031	0.3681	4.0078	7.9790	21.7	62.0	07/07/2011	42	155
CZ6 D R1	02/02/2011	3.6043	0.3694	4.0386	8.0123	21.7	62.3	05/07/2011	26	153
CZ6 D R2	02/02/2011	3.6002	0.3998	4.0104	8.0104	20.0	61.9	28/06/2011	7	146
CZ6 D R3	02/02/2011	3.6030	0.3269	4.5048	8.4347	25.8	66.0	29/06/2011	9	147
CZ7 A R1	03/02/2011	3.6106	0.3362	4.0106	7.9574	23.7	62.3	06/07/2011	36	153
CZ7 D R1	03/02/2011	3.6031	0.3327	4.0061	7.9419	23.9	62.4	05/07/2011	32	152

CZ8 A R1	03/02/2011	3.6013	0.4038	4.0071	8.0122	19.8	61.8	30/06/2011	23	147
CZ8 A R2	03/02/2011	3.6056	0.4595	4.0050	8.0701	17.6	61.3	14/07/2011	49	161
CZ8 A R3	03/02/2011	3.6065	0.4057	4.0124	8.0246	19.8	61.8	06/07/2011	37	153
CZ8 C R1	03/02/2011	3.6049	0.4226	4.0019	8.0294	19.0	61.6	06/07/2011	38	153
CZ8 D R1	03/02/2011	3.6022	0.4013	4.0038	8.0073	20.0	61.8	29/06/2011	16	146
CZ8 D R2	03/02/2011	3.6037	0.4101	4.0082	8.0220	19.6	61.8	30/06/2011	19	147
CZ8 D R3	03/02/2011	3.6035	0.4161	4.0125	8.0321	19.3	61.7	07/07/2011	41	154
CZ9 A R1	04/02/2011	3.5996	0.4051	4.0034	8.0081	19.8	61.8	28/06/2011	1	144
CZ9 A R2	04/02/2011	3.5989	0.3704	4.0059	7.9752	21.5	62.1	28/06/2011	3	144
CZ9 A R3	04/02/2011	3.6079	0.3643	4.0083	7.9805	21.9	62.1	29/06/2011	14	145
CZ9 C R1	04/02/2011	3.6061	0.4268	4.0070	8.0399	18.8	61.6	29/06/2011	15	145
CZ9 C R2	04/02/2011	3.6038	0.4083	4.0120	8.0241	19.7	61.8	28/06/2011	8	144
CZ9 C R3	04/02/2011	3.6096	0.4152	4.0066	8.0314	19.3	61.6	07/07/2011	47	153
CZ9 D R1	04/02/2011	3.6051	0.4016	3.9998	8.0065	19.9	61.8	06/07/2011	35	152
CZ9 D R2	04/02/2011	3.6074	0.4024	4.0335	8.0433	20.0	62.0	06/07/2011	33	152
CZ9 D R3	04/02/2011	3.6050	0.4017	4.0233	8.0300	20.0	61.9	30/06/2011	20	146
CZ10 A R1	04/02/2011	3.6054	0.4014	4.0023	8.0091	20.0	61.8	05/07/2011	31	151
CZ10 A R2	04/02/2011	3.6084	0.4015	4.0070	8.0169	20.0	61.8	29/06/2011	12	145
CZ10 D R1	04/02/2011	3.6065	0.2718	4.0074	7.8857	29.0	62.8	14/07/2011	55	160
Drinking water	04/02/2011	3.6042	0.4064	4.0059	8.0165	19.7	61.7	14/07/2011	52	160
Saliva tube	04/02/2011	3.6105	0.4132	4.0066	8.0303	19.4	61.6	14/07/2011	53	160
MVA sample	14/07/2011		21.5533	21.6039	43.1572		62.1	14/07/2011	54	0

54 samples analysed in total, 51 participant samples. 6 Complete sample sets (A, C & D) participants 1, 2, 5, 6, 8 and 9. Only phases A & D from PP 3, 4, 7 & 10.

Table SI.3.6. Eight blank subtracted elemental drool saliva concentrations (µg.L^{-1}) obtained by ICP-MS from ten participants who participated in the physical stress study

Sample ID	Na	Mg	Si	P	S	K	Zn	I
CZ1 A	16558	733	162	28117	6681	95109	24	7067
CZ1 C	11981	553	80	21043	4759	59161	13	4748
CZ1 D	14957	573	110	24519	4949	1349	21	4150
CZ2 A	15043	442	43	16859	4645	97395	19	1391
CZ2 C	19148	699	76	15767	4953	41386	27	1459
CZ2 D	16964	620	57	18140	4943	1158	18	1739
CZ4 A	17341	490	96	23959	5357	1414	15	4081
CZ4 C	23517	512	55	18954	4221	1615	10	2193
CZ4 D	19787	594	37	22505	4407	112701	15	2158
CZ5 A	48577	2084	205	49322	15347	163077	85	28139
CZ5 C	26946	1004	84	33602	9907	2329	33	11468
CZ5 D	27198	1053	128	24828	7682	2514	44	6645
CZ6 A	6639	290	49	14924	3623	1622	2	3642
CZ6 C	5353	336	60	15295	3631	41444	1	1849
CZ6 D	13963	285	35	14880	3052	1140	6	1460
CZ8 A	16543	382	26	23335	3857	54239	8	1409
CZ8 C	26551	543	51	22794	4553	87787	17	1082
CZ8 D	30617	460	29	22741	3373	41643	12	829
CZ9 A	30745	919	61	24905	8674	32494	20	4784
CZ9 C	40120	1178	103	27871	7764	63829	31	2600
CZ9 D	30680	683	79	15472	4658	60533	30	1761
µ (A)	21635	763	92	25917	6883	63621	25	7216
σ (A)	13828	622	67	11307	4123	58870	28	9434
µ (C)	21945	689	72	22189	5684	42507	19	3628
σ (C)	11265	298	18	6627	2280	31810	12	3656
µ (D)	22024	610	68	20441	4723	31577	21	2678
σ (D)	7315	235	39	4210	1504	43072	13	2033

Table SI.3.7. Urine sample information summary

Sample ID	Sample date	Mass (g)				Urine DF	Amount Ru added ($\mu g.L^{-1}$)	Analysis Date	Analysis Order	No of days in storage
		Sample	2% HNO_3	Internal standard (Ru)	Total					
CZ1 A R1	31/01/2011	0.4927	3.0355	1.0130	4.5412	9.2	56.0	21/09/2011	5	233
CZ1 A R2	31/01/2011	0.3484	3.0067	1.0053	4.3604	12.5	57.8	10/10/2011	62	252
CZ1 A R3	31/01/2011	0.4045	3.0082	1.0068	4.4195	10.9	57.2	10/10/2011	64	252
CZ1 C R1	31/01/2011	0.4643	3.0155	1.0038	4.4836	9.7	56.2	22/09/2011	20	234
CZ1 C R2	31/01/2011	0.4746	3.0018	1.0069	4.4833	9.4	56.4	06/10/2011	59	248
CZ1 C R3	31/01/2011	0.4823	3.0162	1.0172	4.5157	9.4	57.1	01/11/2011	77	274
CZ1 D R1	31/01/2011	0.4549	3.0166	1.0120	4.4835	9.9	56.6	22/09/2011	10	234
CZ1 D R2	31/01/2011	0.1286	3.0046	1.0036	4.1368	32.2	73.6	01/11/2011	80	274
CZ1 D R3	31/01/2011	0.4699	3.0109	1.0052	4.4860	9.5	56.2	06/10/2011	54	248
CZ2 A R1	31/01/2011	0.9426	3.0054	1.0045	4.9525	5.3	50.9	22/09/2011	17	234
CZ2 A R2	31/01/2011	0.9597	3.0040	1.0056	4.9693	5.2	61.4	01/11/2011	84	274
CZ2 A R3	31/01/2011	0.9433	3.0069	1.0053	4.9555	5.3	61.5	01/11/2011	83	274
CZ2 C R1	31/01/2011	0.9129	3.0131	1.0158	4.9418	5.4	51.6	22/09/2011	11	234
CZ2 C R2	31/01/2011	0.9048	3.0166	1.0014	4.9228	5.4	51.0	01/10/2011	38	243
CZ2 C R3	31/01/2011	0.9143	3.0165	1.0023	4.9331	5.4	51.0	01/10/2011	32	243
CZ2 D R1	31/01/2011	0.9675	3.0245	1.0129	5.0049	5.2	50.8	21/09/2011	6	233
CZ2 D R2	31/01/2011	0.9333	3.0040	1.0068	4.9441	5.3	51.1	10/10/2011	63	252
CZ2 D R3	31/01/2011	0.9491	3.0219	1.0037	4.9747	5.2	61.2	01/11/2011	87	274
CZ3 A R1	01/02/2011	1.0162	3.0054	1.0140	5.0356	5.0	50.5	21/09/2011	7	232
CZ3 A R2	01/02/2011	1.0061	3.0118	1.0098	5.0277	5.0	50.9	01/11/2011	78	273
CZ3 A R3	01/02/2011	0.9746	3.0184	1.0034	4.9964	5.1	50.4	06/10/2011	49	247
CZ3 C R1	01/02/2011	0.9757	3.0234	1.0150	5.0141	5.1	50.8	22/09/2011	12	233
CZ3 C R3	01/02/2011	0.9821	3.0116	1.0028	4.9965	5.1	50.4	01/10/2011	46	242
CZ3 C R3	01/02/2011	0.9406	3.0087	1.0081	4.9574	5.3	51.6	01/11/2011	76	273
CZ3 D R1	01/02/2011	0.9457	3.0091	1.0158	4.9706	5.3	51.3	21/09/2011	9	232
CZ3 D R2	01/02/2011	0.9903	3.0078	1.0050	5.0031	5.1	50.4	01/10/2011	48	242
CZ3 D R3	01/02/2011	0.9920	3.0080	1.0060	5.0060	5.0	60.9	01/11/2011	86	273
CZ4 A R1	01/02/2011	0.9292	3.0113	1.0121	4.9526	5.3	51.3	20/09/2011	3	231
CZ4 A R2	01/02/2011	0.9886	3.0217	1.0065	5.0168	5.1	50.3	01/10/2011	40	242
CZ4 A R3	01/02/2011	0.9784	3.0093	1.0053	4.9930	5.1	50.5	06/10/2011	53	247
CZ4 C R1	01/02/2011	0.9630	3.0214	1.0015	4.9859	5.2	50.4	22/09/2011	15	233
CZ4 C R2	01/02/2011	0.9662	3.0087	1.0069	4.9818	5.2	50.7	10/10/2011	71	251

CZ4 C R3	01/02/2011	0.9755	3.0178	1.0016	4.9949	5.1	50.3	01/10/2011	37	242
CZ4 D R1	01/02/2011	0.9364	3.0058	1.0015	4.9437	5.3	50.8	01/10/2011	25	242
CZ4 D R2	01/02/2011	0.9489	3.0070	1.0072	4.9631	5.2	50.9	10/10/2011	72	251
CZ4 D R3	01/02/2011	0.9108	3.0007	1.0072	4.9187	5.4	51.4	10/10/2011	69	251
CZ5 A R1	02/02/2011	0.9419	3.0140	1.0062	4.9621	5.3	50.9	01/10/2011	29	241
CZ5 A R2	02/02/2011	0.9798	3.0089	1.0103	4.9990	5.1	50.7	10/10/2011	65	250
CZ5 A R3	02/02/2011	0.9683	3.0167	1.0198	5.0048	5.2	51.1	10/10/2011	67	250
CZ5 C R1	02/02/2011	0.9231	3.0155	1.0014	4.9400	5.4	50.9	01/10/2011	26	241
CZ5 C R2	02/02/2011	0.9585	3.0155	1.0065	4.9805	5.2	50.7	01/10/2011	35	241
CZ5 C R3	02/02/2011	0.9874	3.0184	1.0113	5.0171	5.1	50.6	10/10/2011	68	250
CZ5 D R1	02/02/2011	0.4372	3.0054	1.0001	4.4427	10.2	56.5	22/09/2011	14	232
CZ5 D R2	02/02/2011	0.9723	3.0003	1.0087	4.9813	5.1	61.4	01/11/2011	79	272
CZ5 D R3	02/02/2011	0.9903	3.0196	1.0024	5.0123	5.1	50.2	01/10/2011	42	241
CZ6 A R1	02/02/2011	0.9331	3.0017	1.0134	4.9482	5.3	51.4	21/09/2011	8	231
CZ6 A R2	02/02/2011	0.9986	3.0155	1.0055	5.0196	5.0	50.8	01/11/2011	73	272
CZ6 A R3	02/02/2011	1.0001	3.0063	1.0006	5.0070	5.0	50.1	01/10/2011	45	241
CZ6 C R1	02/02/2011	1.0197	3.0097	1.0005	5.0299	4.9	49.9	22/09/2011	13	232
CZ6 C R2	02/02/2011	1.0120	3.0050	1.0075	5.0245	5.0	50.9	01/11/2011	74	272
CZ6 C R3	02/02/2011	0.9907	3.0058	1.0062	5.0027	5.0	50.5	06/10/2011	60	246
CZ6 D R1	02/02/2011	0.8872	0.0153	1.0038	1.9063	2.1	132.1	22/09/2011	18	232
CZ6 D R2	02/02/2011	0.9751	3.0025	1.0069	4.9845	5.1	50.7	06/10/2011	58	246
CZ6 D R3	02/02/2011	0.9593	3.0079	1.0059	4.9731	5.2	50.8	01/10/2011	33	241
CZ7 A R1	03/02/2011	0.9742	3.0180	1.0154	5.0076	5.1	50.9	20/09/2011	1	229
CZ7 A R2	03/02/2011	0.9877	3.0166	1.0060	5.0103	5.1	50.4	06/10/2011	56	245
CZ7 A R3	03/02/2011	1.0078	3.0045	1.0037	5.0160	5.0	60.7	01/11/2011	88	271
CZ7 C R1	03/02/2011	0.9370	3.0015	1.0149	4.9534	5.3	51.4	20/09/2011	2	229
CZ7 C R2	03/02/2011	0.9337	3.0044	1.0074	4.9455	5.3	61.8	01/11/2011	81	271
CZ7 C R3	03/02/2011	0.9974	3.0032	1.0054	5.0060	5.0	60.9	01/11/2011	85	271
CZ7 D R1	03/02/2011	0.9321	3.0113	1.0050	4.9484	5.3	51.0	22/09/2011	21	231
CZ7 D R2	03/02/2011	0.9618	3.0078	1.0030	4.9726	5.2	50.6	01/10/2011	47	240
CZ7 D R3	03/02/2011	0.9535	3.0146	1.0047	4.9728	5.2	50.7	06/10/2011	52	245
CZ8 A R1	03/02/2011	0.9247	3.0060	1.0133	4.9440	5.3	51.4	20/09/2011	4	229
CZ8 A R2	03/02/2011	0.9703	3.0113	1.0042	4.9858	5.1	50.5	01/10/2011	44	240
CZ8 A R3	03/02/2011	0.9731	3.0041	1.0066	4.9838	5.1	50.7	06/10/2011	51	245
CZ8 C R1	03/02/2011	0.9817	3.0210	1.0005	5.0032	5.1	50.2	22/09/2011	16	231
CZ8 C R2	03/02/2011	1.0023	3.0209	1.0053	5.0285	5.0	50.2	01/10/2011	36	240

CZ8 C R3	03/02/2011	1.0019	3.0054	1.0081	5.0154	5.0	50.4	06/10/2011	50	245
CZ8 D R1	03/02/2011	0.9527	3.0105	1.0043	4.9675	5.2	50.7	01/10/2011	30	240
CZ9 A R1	04/02/2011	0.9757	3.0088	1.0001	4.9846	5.1	50.3	01/10/2011	27	239
CZ9 A R2	04/02/2011	1.0114	3.0123	1.0049	5.0286	5.0	60.6	01/11/2011	82	270
CZ9 A R3	04/02/2011	0.9867	3.0177	1.0061	5.0105	5.1	50.4	01/10/2011	34	239
CZ9 C R1	04/02/2011	0.9995	3.0285	1.0010	5.0290	5.0	49.9	01/10/2011	28	239
CZ9 C R2	04/02/2011	0.9774	3.0125	1.0006	4.9905	5.1	50.3	01/10/2011	41	239
CZ9 C R3	04/02/2011	0.9762	3.0027	1.0072	4.9861	5.1	50.7	10/10/2011	66	248
CZ9 D R1	04/02/2011	0.9401	3.0225	1.0051	4.9677	5.3	50.8	22/09/2011	24	230
CZ9 D R2	04/02/2011	0.9933	3.0227	1.0007	5.0167	5.1	50.0	01/10/2011	43	239
CZ9 D R3	04/02/2011	0.9489	3.0058	1.0081	4.9628	5.2	51.0	10/10/2011	70	248
CZ10 A R1	04/02/2011	0.9255	3.0038	1.0039	4.9332	5.3	51.1	22/09/2011	19	230
CZ10 A R2	04/02/2011	0.9746	3.0108	1.0060	4.9914	5.1	50.6	06/10/2011	57	244
CZ10 A R3	04/02/2011	0.8591	3.0253	1.0061	4.8905	5.7	51.6	01/10/2011	39	239
CZ10 C R1	04/02/2011	0.9081	3.0069	1.0068	4.9218	5.4	51.3	22/09/2011	22	230
CZ10 C R2	04/02/2011	0.9850	3.0048	1.0076	4.9974	5.1	50.6	10/10/2011	61	248
CZ10 C R3	04/02/2011	1.0060	3.0065	1.0098	5.0223	5.0	51.0	01/11/2011	75	270
CZ10 D R1	04/02/2011	0.9527	3.0117	1.0052	4.9696	5.2	50.8	22/09/2011	23	230
CZ10 D R2	04/02/2011	0.9135	3.0066	1.0047	4.9248	5.4	51.2	01/10/2011	31	239
CZ10 D R3	04/02/2011	0.9834	3.0151	1.0067	5.0052	5.1	50.5	06/10/2011	55	244
MVA Sample	12/10/2011							12/10/2011	89	0
Pot 1	12/10/2011	1.0135	3.0056	1.0058	5.0249	5.0	60.7	12/10/2011	90	0
Pot 2	12/10/2011	1.0040	3.0155	1.0040	5.0235	5.0	60.6	12/10/2011	91	0

Table SI.3.8. Eight blank subtracted elemental urine concentrations (µg.L^{-1}) obtained by ICP-MS from ten participants who participated in the physical stress study

Sample ID	Mg	Si	P	S	K	Ca	Zn	Mo
CZ1 A	179295	12623	1117704	1581731	4395455	148703	813	86
CZ1 C	85180	5638	204822	957477	2960584	63733	258	0
CZ1 D	153876	14329	296812	1479541	5114787	84535	881	0
CZ2 A	7252	1087	61027	258579	1440865	7182	0	0
CZ2 C	11139	2278	84250	287281	1889069	10383	122	0
CZ2 D	40592	6970	371088	683235	2729142	22087	298	46
CZ3 A	104652	3802	1166407	848603	1809429	47414	222	19
CZ3 C	16131	3013	133741	648143	2750812	30078	0	0
CZ3 D	201593	18601	1410752	3990159	9417103	88497	518	0
CZ4 A	95916	8118	1262844	949861	3251645	36403	479	0
CZ4 C	12457	3129	241458	189696	344388	5408	0	9
CZ4 D	83889	13629	843747	697082	1714648	15270	325	58
CZ5 A	550888	9441	4480589	2840938	8660429	93027	694	51
CZ5 C	252574	8889	1451993	1538030	7142834	46785	481	33
CZ5 D	394678	11840	1507232	1727938	6002618	48491	691	0
CZ6 A	6693	3841	261781	443338	2865846	22090	0	41
CZ6 C	2793	2324	44251	240950	1357473	13179	0	0
CZ6 D	4966	4836	130981	516586	2085060	18136	24	27
CZ7 A	185174	28313	2099510	1869746	2878737	91801	933	284
CZ7 C	130103	25516	1022245	1593391	3529179	89183	627	225
CZ7 D	652941	51594	2412782	3380679	6136854	90445	1236	78
CZ8 A	70110	2733	54322	430711	2587045	13039	0	28
CZ8 C	60399	2879	34788	445870	2837930	9225	0	28
CZ8 D	837247	18879	1678989	3721293	7870350	49241	864	105
CZ9 A	522804	11671	3340923	2892711	7342667	50939	354	86
CZ9 C	218982	7742	140664	1177725	2878264	37604	175	35
CZ9 D	751947	16805	224746	3163296	7774028	143091	637	0
CZ10 A	129582	5455	592205	1348902	4500254	18262	207	56
CZ10 C	122515	3824	635539	1162582	4274951	18151	164	74
CZ10 D	242608	13968	1573415	2219290	6415545	34339	746	66
µ (Phase A)	185237	8709	1443731	1346512	3973237	52886	370	65
σ (Phase A)	194955	7898	1469400	954312	2350893	45259	348	83
µ (Phase C)	91227	6523	399375	824114	2996548	32373	183	40
σ (Phase C)	89387	7061	484237	533712	1830530	27423	218	69
µ (Phase D)	336434	17145	1045054	2157910	5526014	59413	622	38
σ (Phase D)	307050	12934	779039	1331612	2612786	41284	347	38

Table SI.3.9. Plasma sample information summary

Sample ID	Sample date	Mass (g)			Plasma DF	Analysis date	Analysis Order	No of days in storage
		Sample	0.5% HNO_3	Total				
CZ1 A	31/01/2011	0.1973	9.8144	10.0117	50.7	12/07/2012	1	528
CZ1 C	31/01/2011	0.2014	9.7991	10.0005	49.7	13/07/2012	19	529
CZ1 D	31/01/2011	0.2051	9.7971	10.0022	48.8	12/07/2012	3	528
CZ2 A	31/01/2011	0.2161	9.8116	10.0277	46.4	12/07/2012	12	528
CZ2 C	31/01/2011	0.2008	9.8142	10.0150	49.9	12/07/2012	9	528
CZ2 D	31/01/2011	0.2023	9.8043	10.0066	49.5	13/07/2012	15	529
CZ3 A	01/02/2011	0.2006	9.8009	10.0015	49.9	13/07/2012	17	528
CZ3 C	01/02/2011	0.2009	9.8053	10.0062	49.8	12/07/2012	4	527
CZ3 D	01/02/2011	0.2022	9.7990	10.0012	49.5	13/07/2012	16	528
CZ4 A	01/02/2011	0.1998	9.8103	10.0101	50.1	12/07/2012	2	527
CZ4 C	01/02/2011	0.2015	9.8026	10.0041	49.6	13/07/2012	23	528
CZ4 D	01/02/2011	0.2019	9.8034	10.0053	49.6	13/07/2012	22	528
CZ5 A	02/02/2011	0.2005	9.8062	10.0067	49.9	12/07/2012	11	526
CZ5 C	02/02/2011	0.2014	9.8088	10.0102	49.7	13/07/2012	14	527
CZ5 D	02/02/2011	0.2021	9.7988	10.0009	49.5	12/07/2012	5	526
CZ6 A	02/02/2011	0.2021	9.8049	10.0070	49.5	13/07/2012	20	527
CZ6 C	02/02/2011	0.2021	9.8096	10.0117	49.5	13/07/2012	18	527
CZ6 D	02/02/2011	0.2022	9.8002	10.0024	49.5	12/07/2012	6	526
CZ7 A	03/02/2011	0.2013	9.8072	10.0085	49.7	12/07/2012	7	525
CZ7 C	03/02/2011	0.2037	9.8083	10.0120	49.2	13/07/2012	13	526
CZ7 D	03/02/2011	0.2019	9.8028	10.0047	49.6	12/07/2012	8	525
2% HNO_3	12/07/2012					12/07/2012	10	0
DI H_2O	12/07/2012					13/07/2012	21	1
QC sample	13/07/2012	1.0000	49.0093	50.0093	50.0			

Table SI.3.10. Seven blank subtracted elemental plasma (venous blood) concentrations ($\mu g.L^{-1}$) obtained by ICP-MS from ten participants who participated in the physical stress study

Sample ID	Li	Mg	P	K	Fe	Cu	Zn
CZ1 A	4720	14226	118367	169618	930	775	1264
CZ1 C	3062	12062	80484	57857	724	498	830
CZ1 D	6784	14650	112908	184314	944	777	1228
CZ2 A	4631	12403	90094	125720	583	628	1060
CZ2 C	4522	12823	99118	141756	753	699	1220
CZ2 D	1752	11510	63522	116765	462	433	689
CZ3 A	2421	11044	70939	102129	537	471	522
CZ3 C	6759	14563	118182	166764	957	891	1023
CZ3 D	2873	10727	70756	116795	651	491	552
CZ4 A	5872	14598	105918	170695	1265	895	992
CZ4 C	3593	12110	78707	108212	755	594	605
CZ4 D	2109	12138	79308	123012	892	609	637
CZ5 A	2313	11314	104330	128570	883	742	1013
CZ5 C	2941	11748	87324	131092	979	601	776
CZ5 D	4666	12297	105158	165827	1140	813	1028
CZ6 A	3200	13542	84998	104907	896	541	541
CZ6 C	1937	10362	63123	111630	777	424	419
CZ6 D	3535	12774	94119	153198	1131	708	756
CZ7 A	4870	13244	89771	139885	458	727	842
CZ7 C	3159	13873	78298	118806	688	593	679
CZ7 D	4357	12194	78735	142270	624	711	807
μ (Phase A)	4004	12910	94917	134503	793	683	891
σ (Phase A)	1364	1377	15719	27714	284	146	275
μ (Phase C)	3711	12506	86462	119445	805	614	793
σ (Phase C)	1551	1398	17675	33797	115	150	266
μ (Phase D)	3725	12327	86358	143169	835	649	814
σ (Phase D)	1728	1216	18218	26162	262	144	237

4 A study into the effect of emotion stress on the elemental content of saliva as measured by ICP-MS

Table SI.4.1. Sample information summary for the elemental profiling of whole saliva, by ICP-MS, from emotional stress and sleep determination

Sample ID	Sample Date	Sample Day	Pre ICP-MS dilution				Dilution Factor	Analysis Date	Analysis Order	No. of days in storage
			whole saliva (g)		2% HNO_3 (g)					
			μ (n=3)	σ (n=3)	μ (n=3)	σ (n=3)				
Pre A	09/11/2011	1	0.9230	0.0002	4.0148	0.0001	5.35	07/12/2011	6	28
Pre B	09/11/2011	1	0.9373	0.0002	4.0020	0.0002	5.27	07/12/2011	1	28
Pre C	09/11/2011	1	0.9333	0.0002	4.0038	0.0001	5.29	07/12/2011	7	28
Post A	10/11/2011	2	1.0396	0.0002	4.0148	0.0001	4.86	07/12/2011	4	27
Post B	10/11/2011	2	1.3866	0.3580	4.0055	0.0001	3.89	07/12/2011	2	27
Post C	10/11/2011	2	1.0819	0.0001	4.0078	0.0001	4.70	07/12/2011	3	27

Table SI.4.2. ICP-MS operating parameters for the analysis of saliva

Entry	Isotope	Accurate Mass	Settling Time	Sample Time	Samples per Peak	Segment Duration	Scan Type	Detection Mode
Low								
1	^{7}Li	7.0155	0.300	0.01	20	0.10	E Scan	Triple
2	^{11}B	11.0088	0.015	0.01	20	0.10	E Scan	Triple
3	^{23}Na	22.9892	0.030	0.01	50	0.10	E Scan	Triple
4	^{75}As	74.9211	0.064	0.01	50	0.10	E Scan	Triple
5	^{88}Sr	87.9051	0.001	0.01	50	0.10	E Scan	Triple
6	^{95}Mo	94.9053	0.001	0.01	50	0.10	E Scan	Triple
7	^{115}In	114.9033	0.030	0.01	50	0.10	E Scan	Triple
8	^{118}Sn	117.9011	0.001	0.01	50	0.10	E Scan	Triple
9	^{127}I	126.9039	0.001	0.01	50	0.10	E Scan	Triple
10	^{208}Pb	207.9761	0.051	0.01	50	0.10	E Scan	Triple
Medium								
1	^{24}Mg	23.9845	0.300	0.05	20	1.25	E Scan	Triple
2	^{27}Al	26.9810	0.001	0.05	20	1.25	E Scan	Triple
3	^{28}Si	27.9764	0.001	0.05	20	1.25	E Scan	Triple
4	^{31}P	30.9732	0.001	0.05	20	1.25	E Scan	Triple
5	^{32}S	31.9715	0.039	0.05	20	1.25	E Scan	Triple
6	^{44}Ca	43.9549	0.042	0.05	20	1.25	E Scan	Triple
7	^{51}V	50.9434	0.001	0.05	20	1.25	E Scan	Triple
8	^{52}Cr	51.9400	0.001	0.05	20	1.25	E Scan	Triple
9	^{55}Mn	54.9375	0.001	0.05	20	1.25	E Scan	Triple
10	^{56}Fe	55.9344	0.001	0.05	20	1.25	E Scan	Triple
11	^{59}Co	58.9327	0.042	0.05	20	1.25	E Scan	Triple
12	^{60}Ni	59.9302	0.001	0.05	20	1.25	E Scan	Triple
13	^{63}Cu	62.9291	0.001	0.05	20	1.25	E Scan	Triple
14	^{66}Zn	65.9255	0.001	0.05	20	1.25	E Scan	Triple
High								
1	^{39}K	38.9632	0.300	0.20	60	5.00	E Scan	Triple
2	^{78}Se	77.9168	0.066	0.20	60	5.00	E Scan	Triple
3	^{83}Kr	82.9136	0.001	0.20	60	5.00	E Scan	Triple

	[min:sec:ms]	[h:min:sec]	Runs/Passes (Measured):	1*1 + 1*3 + 1*3
Low	00:02:194	00:00:07	Runs/Passes (Evaluated):	1*1 + 1*3 + 1*3
Med.	00:18:633	00:00:56	Res. Switch Delay [s]:	2
High	00:16:067	00:00:48		
	Total time	00:01:55		

In the saliva samples a total of twenty seven elements were scanned with the ICP-MS, ten elements in low resolution, fourteen in medium resolution and three in high resolution. However, as well as being profiled the signal obtained for ^{118}Sn was also used as a correction for ^{115}In signal whilst the signal for ^{83}Kr was used only as a correction for the ^{78}Se signal.

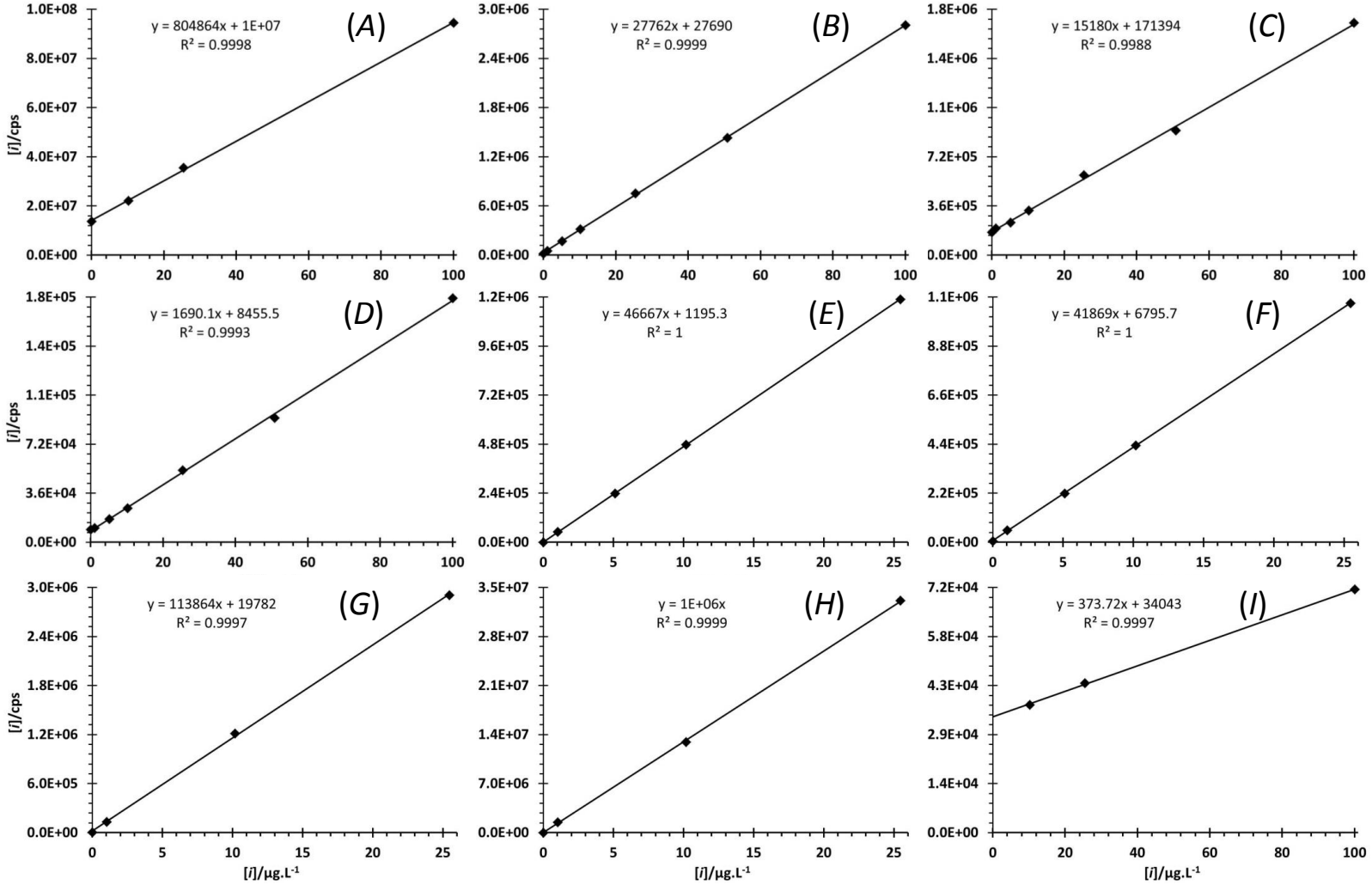


Figure SI.4.1. ICP-MS calibration graphs of (A) sodium (B) magnesium (C) silicon (D) phosphorus (E) manganese (F) iron (G) arsenic (H) strontium and (I) iodine in the standards used for calculating elemental concentrations in whole saliva

5 The Liquid Skin Pen

Table SI.5.1. Sample information summary for Mn recovery testing using the LSP

Sample	ID	Sample Day	Number of samples	Storage	
				Temp (°C)	Duration (days)
EDTA Blank	A	1	1	-20	1
Sample 1	B	1	1	-20	1
Wash 1	B1	1	1	-20	1
Wash 2	B2	1	1	-20	1
Wash 3	B3	1	1	-20	1
DI H_2O Blank	C	1	1	-20	1
HNO_3 Blank	D	1	1	-20	1

Table SI.5.2. Mass (g) of capped delivery vial and solvent (DVS), capped collection vial and solvent (CVS) pre and post sampling of target area using the LSP using three different sample solvents; EDTA; 2% HNO_3; and DI H_2O. S_{DEL} = solvent delivered, S_{COL} = Solvent collected and S_{LOST} = solvent lost, following sample collection, and E = efficiency. n =3

Sample (ID)	DVS							CVS									
	Pre			Post			S_{DEL}*	Pre			Post			S_{COL}*	S_{LOST}**	S_{LOST} (%)	E (%)
	μ	σ	RSD (%)	μ	σ	RSD (%)		μ	σ	RSD (%)	μ	σ	RSD (%)				
EDTA																	
LSP Blank	3.6243	0.0003	0.0073	2.0450	0.0001	0.0028	1.5793	1.9949	0.0001	0.0029	3.5524	0.0001	0.0016	1.5575	0.0218	1.38	98.6
Sample (B)	3.6242	0.0001	0.0016	2.0606	0.0001	0.0049	1.5636	2.0025	0.0001	0.0029	3.5891	0.0001	0.0028	1.5866	-0.0230	-1.47	101.5
Sample wash 1 (B1)	3.5992	0.0005	0.0137	2.0598	0.0001	0.0028	1.5394	1.9902	0.0001	0.0029	3.5178	0.0002	0.0043	1.5276	0.0118	0.77	99.2
Sample wash 2 (B2)	3.6045	0.0001	0.0016	2.0468	0.0001	0.0049	1.5577	1.9537	0.0001	0.0030	3.4981	0.0001	0.0017	1.5445	0.0133	0.85	99.1
Sample wash 3 (B3)	3.6146	0.0001	0.0016	2.0124	0.0002	0.0076	1.6023	2.0039	0.0001	0.0029	3.6203	0.0001	0.0032	1.6165	-0.0142	-0.89	100.9
2% HNO_3																	
LSP Blank	3.5029	0.0002	0.0044	2.0524	0.0001	0.0049	1.4505	1.9892	0.0003	0.0127	3.4737	0.0001	0.0017	1.4845	-0.0340	-2.34	102.3
Sample (B)	3.5923	0.0001	0.0028	2.0693	0.0001	0.0048	1.5230	1.9941	0.0001	0.0058	3.5214	0.0001	0.0028	1.5273	-0.0043	-0.28	100.3
Sample wash 1 (B1)	3.5539	0.0001	0.0016	2.0723	0.0002	0.0074	1.4817	1.9961	0.0001	0.0050	3.4682	0.0001	0.0029	1.4721	0.0096	0.65	99.4
Sample wash 2 (B2)	3.5070	0.0001	0.0016	2.0431	0.0001	0.0028	1.4638	1.9835	0.0001	0.0029	3.4431	0.0002	0.0050	1.4596	0.0043	0.29	99.7
Sample wash 3 (B3)	3.5499	0.0001	0.0028	2.0326	0.0001	0.0028	1.5173	1.9910	0.0001	0.0029	3.5065	0.0001	0.0016	1.5156	0.0017	0.11	99.9
DI H_2O																	
LSP Blank	3.5824	0.0001	0.0028	2.0389	0.0001	0.0028	1.5435	2.0317	0.0001	0.0049	3.5911	0.0001	0.0016	1.5594	-0.0159	-1.03	101.0
Sample (B)	3.4717	0.0001	0.0017	2.0017	0.0001	0.0029	1.4700	2.0105	0.0001	0.0050	3.1600	0.5773	18.2695	1.1495	0.3205	21.80	78.2
Sample wash 1 (B1)	3.6019	0.0001	0.0016	2.0249	0.0001	0.0029	1.5770	1.9939	0.0001	0.0029	3.4673	0.0001	0.0029	1.4734	0.1036	6.57	93.4
Sample wash 2 (B2)	3.6524	0.0001	0.0016	2.0397	0.0001	0.0028	1.6126	1.9584	0.0001	0.0029	3.6828	0.0001	0.0027	1.7244	-0.1118	-6.93	106.9
Sample wash 3 (B3)	3.5312	0.0001	0.0028	2.0270	0.0001	0.0057	1.5042	2.0313	0.0001	0.0049	3.5343	0.0001	0.0016	1.5030	0.0011	0.08	99.9

Table SI.5.3. Participant information summary for the elemental profiling of the volar and plantar

Sample Date	Sample Day	Participant ID	Gender	No of samples	Sample storage
25/07/2011	1	LSP 1	Female	3	-80°C
26/07/2011	2	LSP 2	Male	3	-80°C
27/07/2011	3	LSP 3	Female	3	-80°C
28/07/2011	4	LSP 4	Male	3	-80°C
29/07/2011	5	LSP 5	Female	3	-80°C
01/08/2011	8	LSP 6	Male	3	-80°C
02/08/2011	9	LSP 7	Female	3	-80°C
03/08/2011	10	LSP 8	Male	3	-80°C
04/08/2011	11	LSP 9	Female	3	-80°C
05/07/2011	12	LSP 10	Male	3	-80°C

Table SI.5.4. Sample information summary for the elemental profiling of the volar and plantar

Sample ID	Sample Date	Collection vial mass (g)				Amount of EDTA sampled (g)	Time to collect sample (s)	Pre ICP-MS dilution				Total	Dilution factor	Analysis Date	Analysis Order	No. of day in storage
		Pre		Post				Sample (g)		2% HNO_3 (g)						
		μ (n = 3)	σ (n = 3)	μ (n = 3)	σ (n = 3)			μ (n = 3)	σ (n = 3)	μ (n = 3)	σ (n = 3)					
LSP1 SP Blank	25/07/2011													10/08/2011	4	17
LSP1 E1	25/07/2011	2.2510	0.0000	3.6207	0.0001	1.3697	< 60	1.0010	0.0002	4.0088	0.0001	5.0098	5.0048	10/08/2011	8	17
LSP1 F1	25/07/2011	2.2511	0.0001	3.6238	0.0001	1.3728	< 60	1.0196	0.0001	4.0037	0.0001	5.0233	4.9267	11/08/2011	18	18
LSP2 SP Blank	26/07/2011													10/08/2011	2	16
LSP2 E1	26/07/2011	2.2484	0.0001	3.6588	0.0001	1.4104	< 60	1.0050	0.0001	4.0035	0.0001	5.0086	4.9835	12/08/2011	30	18
LSP2 F1	26/07/2011	2.2534	0.0000	3.6758	0.0001	1.4224	< 60	1.0154	0.0001	4.0084	0.0001	5.0238	4.9477	12/08/2011	27	18
LSP3 SP Blank	27/07/2011													11/08/2011	15	16
LSP3 E1	27/07/2011	1.9703	0.0001	3.4629	0.0001	1.4925	< 60	1.0103	0.0002	4.0008	0.0002	5.0110	4.9601	11/08/2011	16	16
LSP3 F1	27/07/2011	1.9961	0.0001	3.4522	0.0001	1.4561	< 60	1.0082	0.0001	4.0078	0.0001	5.0160	4.9750	11/08/2011	9	16
LSP4 SP Blank	28/07/2011													11/08/2011	13	15
LSP4 E1	28/07/2011	1.9709	0.0001	3.4361	0.0001	1.4652	< 60	1.0034	0.0002	4.0043	0.0003	5.0077	4.9906	10/08/2011	7	14
LSP4 F1	28/07/2011	1.9774	0.0001	3.4848	0.0001	1.5074	< 60	1.0107	0.0001	4.0012	0.0002	5.0120	4.9587	11/08/2011	10	15
LSP5 SP Blank	29/07/2011													11/08/2011	11	13
LSP5 E1	29/07/2011	1.9806	0.0001	3.4795	0.0001	1.4988	< 60	1.0051	0.0001	4.0116	0.0001	5.0167	4.9911	12/08/2011	20	14
LSP5 F1	29/07/2011	2.0116	0.0001	3.5133	0.0001	1.5016	< 60	1.0118	0.0001	4.0014	0.0002	5.0132	4.9547	12/08/2011	21	14
LSP6 SP Blank	01/08/2011													10/08/2011	6	9
LSP6 E1	01/08/2011	2.0088	0.0001	3.3526	0.0001	1.3438	< 60	1.0266	0.0001	4.0132	0.0001	5.0399	4.9091	10/08/2011	3	9
LSP6 F1	01/08/2011	1.9923	0.0001	3.4787	0.0003	1.4864	< 60	1.0120	0.0002	4.0168	0.0001	5.0288	4.9690	10/08/2011	5	9
LSP7 SP Blank	02/08/2011													12/08/2011	26	10
LSP7 E1	02/08/2011	1.9624	0.0001	3.4888	0.0001	1.5264	< 60	1.0170	0.0004	4.0075	0.0001	5.0245	4.9403	12/08/2011	29	10
LSP7 F1	02/08/2011	2.0169	0.0002	3.5085	0.0001	1.4915	< 60	1.0296	0.0002	4.0111	0.0002	5.0406	4.8959	11/08/2011	17	9
LSP8 SP Blank	03/08/2011													12/08/2011	22	9
LSP8 E1	03/08/2011	2.0086	0.0001	3.5126	0.0001	1.5040	< 60	1.0225	0.0001	4.0087	0.0003	5.0312	4.9205	11/08/2011	12	8
LSP8 F1	03/08/2011	2.0197	0.0001	3.4825	0.0001	1.4628	< 60	1.0078	0.0002	4.0071	0.0001	5.0149	4.9761	12/08/2011	24	9
LSP9 SP Blank	04/08/2011													10/08/2011	1	6
LSP9 E1	04/08/2011	1.9738	0.0001	3.4767	0.0001	1.5028	< 60	1.0043	0.0001	4.0084	0.0001	5.0127	4.9914	12/08/2011	19	8
LSP9 F1	04/08/2011	1.9705	0.0001	3.4098	0.0001	1.4393	< 60	1.0045	0.0002	4.0182	0.0001	5.0227	5.0004	12/08/2011	23	8
LSP10 SP Blank	05/07/2011													12/08/2011	28	7
LSP10 E1	05/07/2011	1.9685	0.0002	3.4752	0.0001	1.5066	< 60	1.0130	0.0001	4.0031	0.0001	5.0161	4.9519	11/08/2011	14	6
LSP10 F1	05/07/2011	1.9680	0.0001	3.4656	0.0001	1.4976	< 60	1.0164	0.0002	4.0102	0.0002	5.0267	4.9454	12/08/2011	25	7

Table SI.5.5. Sample information summary for the elemental profiling of the axilla, dorsal, plantar and volar

Sample ID	Sample Date	Collection vial mass (g)				Amount of EDTA sampled (g)	Time to collect sample (s)	Pre ICP-MS dilution				Total	Dilution factor	Analysis Date	Analysis Order	No. of day in storage
		Pre		Post				Sample (g)		2% HNO_3 (g)						
		μ (n = 3)	σ (n = 3)	μ (n = 3)	σ (n = 3)			μ (n = 3)	σ (n = 3)	μ (n = 3)	σ (n = 3)					
U1 AP	29/09/2011	1.9967	0.0001	3.3914	0.0001	1.3948	< 60	1.0062	0.0001	4.0017	0.0001	5.0080	4.98	08/12/2011	1	70
U1 AP Blank	29/09/2011							0.5007	0.0002	4.0666	0.0001	4.5673	9.12	16/12/2011	63	78
U1 TF	29/09/2011	2.0053	0.0001	3.4466	0.0001	1.4413	< 60	1.0125	0.0001	4.0326	0.0155	5.0451	4.98	08/12/2011	15	70
U1 TF Blank	29/09/2011							0.8462	0.0002	4.0178	0.0001	4.8640	5.75	04/01/2012	78	97
U1 BF	29/09/2011	2.0084	0.0001	3.4105	0.0001	1.4021	< 60	1.0216	0.0001	4.0099	0.0001	5.0315	4.92	15/12/2011	31	77
U1 BF Blank	29/09/2011							0.3315	0.0001	4.0017	0.0001	4.3332	13.07	08/12/2011	7	70
U1 AE	29/09/2011	2.0007	0.0001	3.5166	0.0001	1.5159	< 60	1.0261	0.0001	4.0195	0.0001	5.0455	4.92	16/12/2011	51	78
U1 AE Blank	29/09/2011							0.4765	0.0001	4.0121	0.0002	4.4886	9.42	04/01/2012	72	97
U2 AP	29/09/2011	1.9976	0.0001	3.4420	0.0001	1.4444	< 60	1.0085	0.0001	4.0080	0.0001	5.0165	4.97	15/12/2011	42	77
U2 AP Blank	29/09/2011							0.6777	0.0001	4.0276	0.0001	4.7053	6.94	08/12/2011	6	70
U2 TF	29/09/2011	2.0196	0.0001	3.4704	0.0001	1.4508	< 60	1.0245	0.0001	4.0050	0.0001	5.0294	4.91	08/12/2011	16	70
U2 TF Blank	29/09/2011							0.4737	0.0002	4.0009	0.0001	4.4746	9.45	15/12/2011	35	77
U2 BF	29/09/2011	1.9654	0.0001	3.4289	0.0001	1.4634	< 60	1.0141	0.0002	4.0245	0.0002	5.0386	4.97	08/12/2011	8	70
U2 BF Blank	29/09/2011							0.4594	0.0001	4.0053	0.0001	4.4648	9.72	15/12/2011	33	77
U2 AE	29/09/2011	2.0324	0.0001	3.5505	0.0001	1.5181	< 60	1.0149	0.0001	4.0074	0.0001	5.0224	4.95	16/12/2011	69	78
U2 AE Blank	29/09/2011							0.6701	0.0001	4.0000	0.0001	4.6701	6.97	04/01/2012	84	97
U3 AP	29/09/2011	2.0075	0.0000	3.5195	0.0001	1.5120	< 60	1.0120	0.0001	4.0145	0.0001	5.0265	4.97	08/12/2011	5	70
U3 AP Blank	29/09/2011							0.6753	0.0001	4.0153	0.0001	4.6906	6.95	04/01/2012	71	97
U3 TF	29/09/2011	2.0009	0.0001	3.6252	0.0001	1.6243	< 60	1.0087	0.0001	4.0201	0.0002	5.0288	4.99	14/12/2011	30	76
U3 TF Blank	29/09/2011							0.7631	0.0001	4.0088	0.0002	4.7719	6.25	16/12/2011	65	78
U3 BF	29/09/2011	1.9659	0.0001	3.4915	0.0001	1.5257	< 60	1.0209	0.0001	4.0114	0.0001	5.0324	4.93	16/12/2011	70	78
U3 BF Blank	29/09/2011							1.0802	0.0001	4.0258	0.0001	5.1060	4.73	16/12/2011	64	78
U3 AE	29/09/2011	1.9712	0.0000	3.5137	0.0001	1.5425	< 60	1.0152	0.0001	4.0124	0.0002	5.0275	4.95	16/12/2011	55	78
U3 AE Blank	29/09/2011							0.9999	0.0001	4.0106	0.0001	5.0105	5.01	14/12/2011	27	76
U4 AP	29/09/2011	2.0248	0.0001	3.4830	0.0001	1.4582	< 60	1.0169	0.0001	4.0167	0.0001	5.0335	4.95	08/12/2011	11	70
U4 AP Blank	29/09/2011							1.0062	0.0001	4.0120	0.0001	5.0181	4.99	14/12/2011	22	76
U4 TF	29/09/2011	1.9715	0.0001	3.4557	0.0001	1.4842	< 60	1.0186	0.0001	4.0186	0.0001	5.0372	4.95	08/12/2011	19	70
U4 TF Blank	29/09/2011							1.0198	0.0001	4.0192	0.0001	5.0389	4.94	08/12/2011	9	70

U4 BF	29/09/2011	1.9760	0.0001	3.4943	0.0000	1.5183	< 60	1.0112	0.0002	4.0363	0.0001	5.0475	4.99	14/12/2011	28	76
U4 BF Blank	29/09/2011							1.0060	0.0001	4.0221	0.0001	5.0281	5.00	16/12/2011	61	78
U4 AE	29/09/2011	1.9686	0.0001	2.5656	0.0001	0.5970	< 60	0.5868	0.0001	4.0016	0.0001	4.5884	7.82	08/12/2011	13	70
U4 AE Blank	29/09/2011							0.9989	0.0001	4.0270	0.0001	5.0259	5.03	16/12/2011	62	78
U5 AP	29/09/2011	1.9596	0.0001	3.3776	0.0000	1.4180	< 60	0.9993	0.0002	4.0213	0.0000	5.0206	5.02	16/12/2011	58	78
U5 AP Blank	29/09/2011							1.0000	0.0001	4.0034	0.0001	5.0034	5.00	16/12/2011	67	78
U5 TF	29/09/2011	2.0096	0.0000	3.4801	0.0001	1.4705	< 60	1.0146	0.0001	4.0027	0.0001	5.0173	4.95	14/12/2011	23	76
U5 TF Blank	29/09/2011							1.0057	0.0001	4.0041	0.0002	5.0097	4.98	15/12/2011	48	77
U5 BF	29/09/2011	2.0311	0.0001	3.6291	0.0001	1.5980	< 60	1.0141	0.0001	4.0098	0.0001	5.0238	4.95	08/12/2011	10	70
U5 BF Blank	29/09/2011							1.0118	0.0000	4.0090	0.0001	5.0208	4.96	15/12/2011	49	77
U5 AE	29/09/2011	1.9803	0.0001	3.4402	0.0001	1.4599	< 60	1.0148	0.0001	4.0189	0.0001	5.0338	4.96	14/12/2011	26	76
U5 AE Blank	29/09/2011							0.9960	0.0001	4.0123	0.0002	5.0084	5.03	14/12/2011	25	76
U6 AP	30/09/2011	2.0129	0.0001	3.3809	0.0001	1.3680	< 60	1.0051	0.0002	4.0162	0.0001	5.0212	5.00	15/12/2011	41	76
U6 AP Blank	30/09/2011							1.0047	0.0001	4.0152	0.0001	5.0199	5.00	16/12/2011	66	77
U6 TF	30/09/2011	1.9940	0.0001	3.4641	0.0001	1.4701	< 60	1.0006	0.0001	4.0118	0.0001	5.0124	5.01	14/12/2011	24	75
U6 TF Blank	30/09/2011							0.9784	0.0001	4.0108	0.0001	4.9891	5.10	15/12/2011	34	76
U6 BF	30/09/2011	2.0025	0.0001	3.3758	0.0001	1.3733	< 60	1.0143	0.0001	4.0075	0.0001	5.0218	4.95	08/12/2011	17	69
U6 BF Blank	30/09/2011							1.0049	0.0000	4.0102	0.0002	5.0151	4.99	08/12/2011	4	69
U6 AE	30/09/2011	2.0021	0.0001	3.4008	0.0001	1.3987	< 60	1.0180	0.0001	4.0047	0.0001	5.0227	4.93	08/12/2011	3	69
U6 AE Blank	30/09/2011							1.0038	0.0001	4.0216	0.0001	5.0255	5.01	16/12/2011	57	77
U7 AP	30/09/2011	1.9998	0.0001	3.3993	0.0001	1.3995	< 60	1.0116	0.0001	4.0108	0.0002	5.0224	4.96	16/12/2011	59	77
U7 AP Blank	30/09/2011							0.9938	0.0001	4.0173	0.0001	5.0112	5.04	15/12/2011	37	76
U7 TF	30/09/2011	1.9645	0.0001	3.4811	0.0001	1.5166	< 60	1.0115	0.0000	4.0182	0.0002	5.0297	4.97	14/12/2011	21	75
U7 TF Blank	30/09/2011							1.0025	0.0001	4.0238	0.0002	5.0263	5.01	15/12/2011	40	76
U7 BF	30/09/2011	2.0072	0.0001	3.5154	0.0001	1.5083	< 60	1.0126	0.0001	4.0119	0.0001	5.0246	4.96	15/12/2011	36	76
U7 BF Blank	30/09/2011							0.9998	0.0001	4.0042	0.0001	5.0040	5.01	04/01/2012	73	96
U7 AE	30/09/2011	1.9601	0.0001	3.4381	0.0000	1.4780	< 60	1.0158	0.0001	4.0172	0.0002	5.0329	4.95	15/12/2011	47	76
U7 AE Blank	30/09/2011							0.9981	0.0002	4.0830	0.1039	5.0812	5.09	15/12/2011	50	76
U8 AP	30/09/2011	2.0221	0.0001	3.5114	0.0001	1.4894	< 60	1.0082	0.0001	4.0256	0.0001	5.0338	4.99	15/12/2011	43	76
U8 AP Blank	30/09/2011							1.0085	0.0002	4.0262	0.0001	5.0347	4.99	16/12/2011	60	77
U8 TF	30/09/2011	1.9866	0.0001	3.5731	0.0001	1.5866	< 60	1.0169	0.0001	4.0182	0.0002	5.0351	4.95	14/12/2011	29	75
U8 TF Blank	30/09/2011							1.0046	0.0001	4.0160	0.0001	5.0206	5.00	04/01/2012	80	96
U8 BF	30/09/2011	2.0086	0.0001	3.4964	0.0001	1.4878	< 60	1.0223	0.0001	4.0095	0.0001	5.0318	4.92	04/01/2012	81	96

U8 BF Blank	30/09/2011							1.0253	0.0001	4.0183	0.0001	5.0436	4.92	08/12/2011	12	69
U8 AE	30/09/2011	1.9676	0.0001	3.4125	0.0001	1.4449	< 60	1.0174	0.0001	4.0144	0.0002	5.0318	4.95	16/12/2011	53	77
U8 AE Blank	30/09/2011							1.0062	0.0001	4.0166	0.0002	5.0227	4.99	04/01/2012	82	96
U9 AP	30/09/2011	1.9973	0.0001	3.4554	0.0001	1.4581	< 60	1.0119	0.0001	4.0060	0.0001	5.0179	4.96	04/01/2012	75	96
U9 AP Blank	30/09/2011							1.0074	0.0001	4.0157	0.0002	5.0230	4.99	04/01/2012	74	96
U9 TF	30/09/2011	2.0050	0.0000	3.4106	0.0001	1.4056	< 60	1.0081	0.0001	4.0073	0.0001	5.0153	4.98	15/12/2011	44	76
U9 TF Blank	30/09/2011							0.9983	0.0001	4.0149	0.0001	5.0132	5.02	08/12/2011	14	69
U9 BF	30/09/2011	1.9829	0.0001	3.3857	0.0001	1.4028	< 60	1.0202	0.0001	4.0151	0.0001	5.0353	4.94	08/12/2011	20	69
U9 BF Blank	30/09/2011							1.0094	0.0003	4.0101	0.0001	5.0196	4.97	15/12/2011	32	76
U9 AE	30/09/2011	1.9748	0.0000	3.3454	0.0001	1.3706	< 60	1.0120	0.0001	4.0148	0.0001	5.0268	4.97	08/12/2011	18	69
U9 AE Blank	30/09/2011							1.0085	0.0001	4.0282	0.0001	5.0366	4.99	04/01/2012	83	96
U10 AP	30/09/2011	1.9835	0.0001	3.3126	0.0001	1.3291	< 60	1.0155	0.0002	4.0057	0.0003	5.0212	4.94	04/01/2012	77	96
U10 AP Blank	30/09/2011							1.0026	0.0001	4.0137	0.0002	5.0162	5.00	04/01/2012	76	96
U10 TF	30/09/2011	1.9979	0.0001	3.5313	0.0001	1.5335	< 60	1.0095	0.0002	4.0106	0.0001	5.0201	4.97	15/12/2011	38	76
U10 TF Blank	30/09/2011							1.0075	0.0001	4.0095	0.0001	5.0171	4.98	16/12/2011	54	77
U10 BF	30/09/2011	2.0039	0.0001	3.3958	0.0001	1.3920	< 60	1.0200	0.0002	4.0078	0.0001	5.0278	4.93	15/12/2011	45	76
U10 BF Blank	30/09/2011							1.0032	0.0001	4.0029	0.0002	5.0061	4.99	16/12/2011	56	77
U10 AE	30/09/2011	1.9989	0.0001	3.4578	0.0000	1.4589	< 60	1.0216	0.0001	4.0093	0.0001	5.0308	4.92	04/01/2012	79	96
U10 AE Blank	30/09/2011							1.0048	0.0001	4.0092	0.0001	5.0139	4.99	08/12/2011	2	69
EDTA Field Blank	30/09/2011													15/12/2011	39	76
EDTA Blank	30/09/2011													15/12/2011	46	76
2% HNO3 Blank	30/09/2011													16/12/2011	52	77
DI H2O Blank	30/09/2011													17/12/2011	68	78

Table SI.5.6. Participant information summary for the profiling of the dorsal and plantar

Sample Date	Sample Day	Participant ID	Gender	Sample side		No of samples	Sample Storage
				Dorsal	Plantar		
01/06/2012	1	UF1	Male	LEFT FOOT		**5**	-80°C
01/06/2012	1	UF2	Female	RIGHT FOOT		**5**	-80°C
06/06/2012	2	UF3	Male	RIGHT FOOT		**5**	-80°C
06/06/2012	2	UF4	Female	LEFT FOOT		**5**	-80°C
08/06/2012	3	UF5	Male	LEFT FOOT		**5**	-80°C
12/06/2012	4	UF6	Female	RIGHT FOOT		**5**	-80°C
12/06/2012	4	UF7	Male	RIGHT FOOT		**5**	-80°C
13/06/2012	5	UF8	Female	LEFT FOOT		**5**	-80°C
13/06/2012	5	UF9	Male	LEFT FOOT		**5**	-80°C
13/06/2012	5	UF10	Female	RIGHT FOOT		**5**	-80°C

Table SI.5.7. Sample information summary for the profiling of the dorsal and plantar

Sample ID	Sample date	Collection vial mass (g)				Amount of EtOH sampled (g)	Time to collect sample (s)	ES-		ES+			No. of days in storage
		Pre		Post				Analysis date	Analysis order*	Analysis date	Analysis date	Analysis order*	
		μ (n=3)	σ (n=3)	μ (n=3)	σ (n=3)								
UF1 FB	01/06/2012	1.9976	0.0001	2.9074	0.0001	**0.9098**	< 60	05/07/2012	29	34	29/10/2012	5	150
UF1 TF B	01/06/2012	1.9989	0.0001	3.0192	0.0001	**1.0203**	< 60	05/07/2012	20	34	29/10/2012	28	150
UF1 TF	01/06/2012	2.0050	0.0001	2.9860	0.0001	**0.9811**	< 60	05/07/2012	40	34	29/10/2012	33	150
UF1 BF B	01/06/2012	2.0018	0.0003	3.1074	0.0001	**1.1056**	< 60	05/07/2012	17	34	29/10/2012	36	150
UF1 BF	01/06/2012	1.9947	0.0002	2.9724	0.0001	**0.9777**	< 60	05/07/2012	38	34	29/10/2012	38	150
UF2 FB	01/06/2012	2.0087	0.0001	3.0327	0.0001	**1.0240**	< 60	05/07/2012	33	34	29/10/2012	37	150
UF2 TF B	01/06/2012	1.9990	0.0001	2.9890	0.0001	**0.9900**	< 60	05/07/2012	45	34	29/10/2012	34	150
UF2 TF	01/06/2012	2.0617	0.0001	3.1177	0.0001	**1.0560**	< 60	05/07/2012	30	34	29/10/2012	27	150
UF2 BF B	01/06/2012	2.0011	0.0001	2.8057	0.0001	**0.8046**	< 60	05/07/2012	42	34	29/10/2012	9	150
UF2 BF	01/06/2012	1.9908	0.0001	3.0395	0.0001	**1.0487**	< 60	05/07/2012	35	34	29/10/2012	7	150
UF3 FB	06/06/2012	2.0563	0.0001	3.0669	0.0002	**1.0106**	< 60	05/07/2012	49	29	29/10/2012	1	145
UF3 TF B	06/06/2012	2.0332	0.0001	2.9395	0.0001	**0.9064**	< 60	05/07/2012	22	29	29/10/2012	50	145
UF3 TF	06/06/2012	2.0576	0.0001	3.0121	0.0001	**0.9545**	< 60	05/07/2012	8	29	29/10/2012	3	145
UF3 BF B	06/06/2012	2.0149	0.0001	2.7536	0.0001	**0.7387**	< 60	05/07/2012	2	29	29/10/2012	10	145
UF3 BF	06/06/2012	1.9971	0.0001	2.9650	0.0001	**0.9680**	< 60	05/07/2012	34	29	29/10/2012	40	145
UF4 FB	06/06/2012	2.0011	0.0001	3.0302	0.0001	**1.0291**	< 60	05/07/2012	51	29	29/10/2012	41	145
UF4 TF B	06/06/2012	2.0342	0.0001	2.7886	0.0001	**0.7544**	< 60	05/07/2012	37	29	29/10/2012	6	145
UF4 TF	06/06/2012	1.9913	0.0001	2.9682	0.0001	**0.9768**	< 60	05/07/2012	1	29	29/10/2012	45	145
UF4 BF B	06/06/2012	1.9995	0.0001	2.7207	0.0001	**0.7212**	< 60	05/07/2012	47	29	29/10/2012	31	145
UF4 BF	06/06/2012	2.0019	0.0001	2.9634	0.0001	**0.9616**	< 60	05/07/2012	6	29	29/10/2012	24	145
UF5 FB	08/06/2012	2.0043	0.0001	2.7046	0.0002	**0.7003**	< 60	05/07/2012	4	27	29/10/2012	26	143
UF5 TF B	08/06/2012	1.9899	0.0001	3.0137	0.0002	**1.0238**	< 60	05/07/2012	36	27	29/10/2012	48	143
UF5 TF	08/06/2012	1.9940	0.0001	2.9996	0.0002	**1.0056**	< 60	05/07/2012	46	27	29/10/2012	20	143

UF5 BF B	08/06/2012	2.0037	0.0001	2.7535	0.0001	**0.7498**	< 60	05/07/2012	5	27	29/10/2012	47	143
UF5 BF	08/06/2012	2.0674	0.0001	3.0168	0.0001	**0.9494**	< 60	05/07/2012	44	27	29/10/2012	35	143
UF6 FB	12/06/2012	2.0589	0.0001	2.9025	0.0001	**0.8437**	< 60	05/07/2012	43	23	29/10/2012	11	139
UF6 TF B	12/06/2012	2.0314	0.0001	3.0971	0.0001	**1.0657**	< 60	05/07/2012	12	23	29/10/2012	13	139
UF6 TF	12/06/2012	2.0023	0.0001	3.0332	0.0001	**1.0308**	< 60	05/07/2012	23	23	29/10/2012	39	139
UF6 BF B	12/06/2012	2.0565	0.0001	2.9565	0.0001	**0.8999**	< 60	05/07/2012	3	23	29/10/2012	12	139
UF6 BF	12/06/2012	2.0354	0.0001	3.0274	0.0001	**0.9919**	< 60	05/07/2012	39	23	29/10/2012	23	139
UF7 FB	12/06/2012	1.9927	0.0004	3.0076	0.0001	**1.0149**	< 60	05/07/2012	41	23	29/10/2012	14	139
UF7 TF B	12/06/2012	2.0588	0.0001	3.1112	0.0001	**1.0524**	< 60	05/07/2012	25	23	29/10/2012	42	139
UF7 TF	12/06/2012	2.0357	0.0001	2.8327	0.0001	**0.7971**	< 60	05/07/2012	21	23	29/10/2012	29	139
UF7 BF B	12/06/2012	1.9805	0.0001	2.9374	0.0001	**0.9569**	< 60	05/07/2012	32	23	29/10/2012	18	139
UF7 BF	12/06/2012	1.9956	0.0001	2.7970	0.0001	**0.8015**	< 60	05/07/2012	9	23	29/10/2012	4	139
UF8 FB	13/06/2012	2.0672	0.0001	2.9540	0.0001	**0.8868**	< 60	05/07/2012	31	22	29/10/2012	16	138
UF8 TF B	13/06/2012	1.9975	0.0000	3.5124	0.0001	**1.5149**	< 60	05/07/2012	27	22	29/10/2012	44	138
UF8 TF	13/06/2012	2.0063	0.0001	3.0048	0.0001	**0.9985**	< 60	05/07/2012	7	22	29/10/2012	49	138
UF8 BF B	13/06/2012	2.0356	0.0001	3.2051	0.0001	**1.1695**	< 60	05/07/2012	19	22	29/10/2012	25	138
UF8 BF	13/06/2012	1.9905	0.0001	2.9297	0.0001	**0.9392**	< 60	05/07/2012	13	22	29/10/2012	22	138
UF9 FB	13/06/2012	2.0630	0.0001	3.1272	0.0001	**1.0642**	< 60	05/07/2012	24	22	29/10/2012	46	138
UF9 TF B	13/06/2012	1.9997	0.0001	3.1234	0.0001	**1.1238**	< 60	05/07/2012	48	22	29/10/2012	21	138
UF9 TF	13/06/2012	2.0023	0.0001	2.9152	0.0001	**0.9129**	< 60	05/07/2012	10	22	29/10/2012	17	138
UF9 BF B	13/06/2012	2.0407	0.0001	3.4768	0.0001	**1.4360**	< 60	05/07/2012	26	22	29/10/2012	30	138
UF9 BF	13/06/2012	2.0309	0.0001	3.1003	0.0001	**1.0695**	< 60	05/07/2012	15	22	29/10/2012	8	138
UF10 FB	13/06/2012	2.0527	0.0001	2.9688	0.0001	**0.9161**	< 60	05/07/2012	16	22	29/10/2012	19	138
UF10 TF B	13/06/2012	1.9833	0.0001	3.4181	0.0001	**1.4348**	< 60	05/07/2012	28	22	29/10/2012	32	138
UF10 TF	13/06/2012	2.0057	0.0001	2.9775	0.0001	**0.9718**	< 60	05/07/2012	50	22	29/10/2012	2	138
UF10 BF B	13/06/2012	1.9816	0.0001	2.9163	0.5774	**0.9347**	< 60	05/07/2012	14	22	29/10/2012	15	138
UF10 BF	13/06/2012	2.0070	0.0001	3.0236	0.0001	**1.0166**	< 60	05/07/2012	18	22	29/10/2012	51	138
EtOH Blank	31/06/2012							05/07/2012	11	35	29/10/2012	43	151