THE EFFECT OF ISOKINETIC EXERCISE ON BIOFLUID MATRICES AS MEASURED BY ^{1}H NMR SPECTROSCOPY

ALEXANDER LYONS

A thesis submitted in partial fulfilment of the requirements of the

School of Health, Sport and Bioscience, University of East London

for the degree of Doctor of Philosophy

2015

DECLARATION

I hereby certify that the work presented within this thesis is the result of my own investigation, except where reference has been made to published literature and where acknowledgment is made for unpublished data. During the course of this research programme I have not been registered or enrolled for another award from any academic or professional institutions.

- Alexander Lyons

School of Health, Sport and Bioscience

University of East London

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ABSTRACT

The biochemical basis of muscle function during stress imposed by isokinetic exercise has mainly focused on measuring muscle proteins (CK and myoglobin) and lactate present in the blood plasma, whereas the range of low-molecular weight metabolites during muscular stress has received limited attention. This study investigated the effect of concentric isokinetic exercise of the knee extensor and flexor muscles (functional to standing, walking and running), at 80% and 40%MVC intensities of equal workloads, on saliva, urine and blood serum matrices. A hypothesis-free approach was employed to mathematically determine, by chemometrics, which untargeted metabolites measured by ^{1}H NMR spectroscopy are altered in response to muscular exercise of this nature.

The results demonstrate that the saliva matrix is highly susceptible to ^{1}H NMR spectral interference from salivette exposure (a routine method of saliva collection in sports/exercise) with ^{1}H resonances arising throughout the spectrum, exceeding the affected spectral region of δ 3-4 documented in the literature. Short-chain organic acids (lactate, acetate, *n*-butyrate and formate) and *N*-acetyl sugars were the strongest discriminators of saliva sampled pre- and post-exercise. However, the origin of these metabolites is equivocal since bacterial metabolism and salivary gland secretion via exercise stimulation of the autonomic nervous system both contribute towards salivary composition. Urinary citrate, glycine and hippurate exhibited a decrease following isokinetic exercise, whereas trimethylamine *N*-oxide increased. According to the literature, this pattern has previously been associated with kidney stress on the renal papilla and tubules from the filtration of myoglobin protein from the blood which accumulates as a result of leakage from the muscle membrane during stress or injury. Thus, these urinary biomolecular markers may reflect renal filtration of muscle proteins present in the blood following exercise and may be indirectly representative of isokinetic exercise-induced muscular stress. The serum matrix was less sensitive to exercise-induced change than urine in this isokinetic intervention study, since the homeostasis of blood is maintained by the renal system.

Further work is needed to cross-validate conventional assays used to measure biomarkers of muscular stress with the metabolomics platform to confirm that urinary trimethylamine *N*-oxide, citrate, glycine and hippurate are indirect biomolecular markers of muscular exercise. In sports medicine, NMR-urinalysis may provide diagnostic and prognostic information on injured and recovering athletes to determine whether or not they are fit to play, and predict risk of injury based on their current state of health.

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1D	1 Dimensional
^{1}H NMR	Proton Nuclear Magnetic Resonance (Spectroscopy)
ACSM	American College of Sports Medicine
Acetyl CoA	Acetyl Coenzyme A
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CK	Creatine kinase
COPD	Chronic Obstructive Pulmonary Disease
CPMG	Carr-Purcell-Meiboom-Gill
BCAA	Branched-chain amino acids
dL	Decilitre
D_2O	Deuterium oxide
FID/FIDs	Free induction decay/s
g	Grams
g	Centrifuge speed
GC-MS	Gas chromatography – mass spectrometry
HIE	High Intensity Exercise (intervention in this study)
hr	Hours
HMDB	Human Metabolome Database (www.hmdb.ca)
Hz	Hertz
IgA	Immunoglobin A
kDa	KiloDaltons
K	Kelvin temperature
LDL	Low density lipoproteins
LIE	Low Intensity Exercise (intervention in this study)
L	Litre
LC-MS	Liquid chromatography – mass spectrometry
M	Molar
MCTs	Monocarboxylate transporters
mg	Milligrams
min	Minutes

mL	Millilitre
mM	Millimolar
ms	Milliseconds
MVC	Maximal voluntary contraction
MHz	Megahertz
N	Newtons
***n* =**	Number of participants
NOESY	Nuclear Overhauser Effect Spectroscopy
PARQ	Pre-exercise Readiness Questionnaire
PC1/PC2	Principal Component 1/Principal Component 2, respectively
PCA	Principal Component Analysis
PLS-DA	Partial Least Squares-Discrimination Analysis
ppm	Parts per million
ROS	Reactive oxygen species
s	Seconds
SD	Standard Deviation
T_1	Longitudinal relaxation (Spin-lattice)
T_2	Transverse relaxation (spin-spin)
TCA Cycle	Tricarboxylic Acid Cycle
TMAO	Trimethylamine *N*-Oxide
TSP	Trimethylsilyl-propionate
UEL	University of East London
UREC	University of East London's Research Ethics Committee
UMDB	Urine Metabolome Database (www.urinemetabolome.ca)
µg	Micrograms
µL	Microlitres
µM	Micromolar concentration
µs	Microseconds
VLDL	Very low density lipoproteins
δ	Chemical Shift (ppm)

ACKNOWLEDGMENTS

I thank my Director of Studies, Professor Olivia Corcoran, for her drive and enthusiasm towards my PhD research and for her support, technical discussions and encouragement throughout this venture. Olivia sure has a unique and challenging way of asking the right questions, without directly giving away the answers, which guided me throughout this independent research programme. I thank my supervisory team, Dr Roger Carpenter and Dr Jane Culpan for their encouragement, faith and support in overcoming the challenges faced as a PhD student. I am grateful to the Medicines Research Group (MRG) and the Human Motor Performance Group (HMPG), School of Health, Sport and Bioscience at the University of East London for the fully-funded PhD scholarship.

I sincerely thank my collaborators Dr Harold Toms, NMR spectroscopy manager at Queen Mary University London and Dr Anthony Dona, former NMR manager at Imperial College London for their time and patience in training me to operate Bruker NMR instruments to acquire the metabolomic data for this project. Harold kindly allocated extra time on several occasions for me to accompany him and learn more about the operations and theory underpinning this fascinating instrument. Thank you to Prof. Martin Grootveld and his PhD student Victor at De Montfort University for their advice, training and use of the 400 MHz NMR spectrometer. I thank Dr Calvin Moorley and Kevin Clough for their supervision during my phlebotomy course, which permitted me to collect venous blood samples from the volunteers, to whom I am grateful for their participation, in this project.

My gratitude extends to my partner Alessia for her patience, comfort, encouragement and confidence in me, and always being available to listen to me and help put problems into perspective. I thank my parents for always prioritising my career decisions and aspirations over the financial income, and my friends/peer PhD students for their advice.

CHAPTER 1: INTRODUCTION

1.1 Muscle Function and Isokinetic Exercise

The functional capacity of skeletal muscle underpins the ability to carry out everyday tasks and may have implications for sports medicine and rehabilitation in terms of performance. Muscle function refers to the force produced throughout the range of motion of the joint, which may be assessed by dynamometry and enhanced by resistance-based isokinetic exercise at set velocities (Caruso *et al.*, 2012). Isokinetic dynamometry has the advantage of mechanically controlling the range of motion, angular-velocity and type of muscle contraction performed by providing a counter-torque dependent on the force applied by the individual (Caruso *et al.*, 2012). This ability to adjust, control and measure such parameters has facilitated the study of force-velocity (Maffiuletti *et al.*, 2007) and joint angular-force relationships (Child *et al.*, 1998), and muscle function of concentric-specific and/or eccentric-specific contractions (Child *et al.*, 1998; Overend *et al.*, 2000; Marzorati *et al.*, 2000; Paschalis *et al.*, 2005; Maffiuletti *et al.*, 2007; Speranza *et al.*, 2007; Rainoldi *et al.*, 2008; Knoblauch *et al.*, 2010; Lee and Kang, 2013; Margaritelis *et al.*, 2014). In addition to the force output data retrievable from the dynamometer, data has been published on cardiovascular and/or muscular stress imposed by isokinetic exercise (Child *et al.*, 1998; Marzorati *et al.*, 2000; Overend *et al.*, 2000; Paschalis *et al.*, 2005; Speranza *et al.*, 2007; Rainoldi *et al.*, 2008; Knoblauch *et al.*, 2010; Margaritelis *et al.*, 2014). In these studies, cardiovascular stress was determined by heart rate and blood pressure measurements, whereas muscular stress was determined by the analysis of biomolecular markers.

The concentration of plasma creatine kinase (CK), an enzyme that facilitates ATP and ADP buffering, has been reported to significantly increase after eccentric isokinetic exercise (Child *et al.*, 1998; Paschalis *et al.*, 2005; Knoblauch *et al.*, 2010) but not after concentric isokinetic exercise (Speranza *et al.*, 2007). Briefly, eccentric and concentric

actions refer to the lengthening and shortening phases, respectively, of muscle contractions. The association between eccentric exercise and muscle micro-trauma has been comprehensively reviewed previously (Nikolaidis *et al.*, 2008; Brancaccio *et al.*, 2010; Brentano and Martins Kruel, 2011). The mechanism underpinning micro-trauma of muscle tissue originates from degeneration of the sarcomere (functional units of muscle) via Z-disk fragmentation from overstretching (Brancaccio *et al.*, 2010), hence the link to eccentric exercise. By contrast, Speranza *et al.* (2007) reported a marked increase in blood plasma myoglobin concentration following maximal contractions during concentric isokinetic exercise, which is indicative of muscle protein leakage into the blood stream from the local site of swelling or trauma to the muscle membrane (Nikolaidis *et al.*, 2008; Brancaccio *et al.*, 2010; Brentano and Martins Kruel, 2011).

Other plasma biomolecular markers of muscular stress measured in isokinetic studies include the stress hormone cortisol (Rainoldi *et al.*, 2008), the markers of purine metabolism hypoxanthine and xanthine (Speranza *et al.*, 2007), and reactive oxygen species (indicative of oxidative stress) including protein carbonyls and erythrocyte glutathione, and urinary isoprostanes (Margaritelis *et al.*, 2014). Such biomolecular data may provide diagnostic and prognostic information on the return to normal function of injured and recovering individuals, and offer efficacy data on resistance-based training interventions. However, to the author's knowledge, isokinetic exercise studies have received limited attention on the profiling of low-molecular weight metabolites (≤ 1 kDa) to, potentially, further elucidate the biochemistry associated with muscular stress during exercise and thus, metabolomics may be a promising approach to serve this purpose, as discussed in Section 1.2.

To carry out a metabolomics study on isokinetic exercise, it is necessary to first consider the modality, velocity, range of motion, intensity and volume parameters to employ. In muscle function studies, isokinetic knee extension and/or flexion exercise has been well researched (Bond *et al.*, 1991; Child *et al.*, 1998; Overend *et al.*, 2000; Marzorati *et al.*,

2000; Paschalis *et al.*, 2005; Maffiuletti *et al.*, 2007; Speranza *et al.*, 2007; Rainoldi *et al.*, 2008; Margaritelis *et al.*, 2014), possibly because the muscles of the thigh (Table 1.1) are of a relatively large mass with a rich blood supply and are functional to daily activities such as standing, walking and running. Thus, isokinetic exercise of the knee extensor and flexor muscles may elicit a greater metabolic response than upper-body exercise. In addition, less intra-group variability in force output data was reported in studies that utilised lower-body isokinetic exercise compared to upper-body, possibly because the design of dynamometers are best suited for knee extension and flexion movements, according to a recent review (Caruso *et al.*, 2012). In previous studies (Bond *et al.*, 1991; Child *et al.*, 1998; Overend *et al.*, 2000; Marzorati *et al.*, 2000; Paschalis *et al.*, 2005; Maffiuletti *et al.*, 2007; Speranza *et al.*, 2007), the angular velocities employed for knee extension and/or flexion exercise ranged from 30-180°.s^{-1}, with higher force production reported at the lower range, and 60°.s^{-1} being the most common amongst these studies. To generate muscular force through a full range of motion, it is reasonable to employ the maximum range possible; the dynamometer supports a range of motion between 90° of knee flexion to full extension (Caruso *et al.*, 2012).

The effect of equal volumes of high and low intensity eccentric isokinetic exercise on muscle micro-trauma (as indicated by plasma CK concentrations) and performance (peak torque generated) has been investigated previously (Paschalis *et al.*, 2005). The study reported a similar response in plasma CK elevations between the two intensities and greater performance attenuation after high intensity exercise, which suggests that muscle micro-trauma is most influenced by the standardised volume as opposed to exercise intensity, in the context of eccentric loading. However, to the author's knowledge, the metabolic consequences of concentric isokinetic exercise of the knee extensor and flexor muscles at high and low intensities at a standardised workload have yet to be investigated. A metabolomics investigation on these isokinetic parameters may further elucidate low-molecular weight biomarkers of muscle stress during concentric loading.

Table 1.1 Muscles responsible for knee extension and flexion, according to Tortora and Derrickson (2005)

Knee Extensors			Knee Flexors		
Muscle	**Origin**	**Insertion**	**Muscle**	**Origin**	**Insertion**
Rectus Femoris	• Anterior inferior iliac spine of ilium; groove above acetabulum	• Superior aspect of patella; patella tendon to tibial tuberosity	**Biceps Femoris**	• Ischial tuberosity (Long Head) • Linea aspera and lateral condyloid ridge (Short Head)	• Lateral condyle of tibia; head of fibula; deep fascia on lateral side of the thigh
Vastus Intermedius	• Upper-two thirds of anterior surface of femur	• Upper boarder aspect of patella; patella tendon to tibial tuberosity	**Semitendinosus**	• Ischial tuberosity	• Anterior medial surface of tibia below condyle
Vastus Medialis	• Entire length of linea aspera; medial condyloid ridge	• Medial half of aspect of patella; patella tendon to tibial tuberosity	**Semimembranosus**	• Ischial tuberosity	• Posteromedial surface of medial tibial condyle
Vastus Lateralis	• Greater trochanter; linea aspera	• Lateral border of patella; patella tendon to tibial tuberosity	**Sartorius**	• Anterior superior iliac spine • Notch below spine	• Anterior medial surface of tibia below the condyle
Tensor Fasciae Latae	• Anterior part of outer iliac crest; outer surface of ilium below crest	• One-fourth of the ilitibialband which inserts into Gerdy's tubercle on anterolateral tibial condyle	**Gracilis**	• Anterior aspect of lower half of pubic symphasis; medial margin of inferior ramus of pubis	• Anterior medial surface of tibia below condyle
			Popliteus	• Posterior surface of lateral condyle of femur	• Upper posterior medial surface of tibia
			Gastrocnemius	• Posterior surface of medial condyle of femur (Medial head) • Posterior surface of lateral condyle of femur (Lateral head)	

1.2 Metabolomics in Exercise Physiology

Metabolic profiling of biological samples provides a snapshot of data that can be informative of the physiological and biochemical state of an individual and can differentiate between populations based on pathophysiology, for example, severity of hypertension in black and white Americans (Stamler *et al.*, 2013). Fluctuations and regulation of biological systems are also measurable through metabolic profiling, via making comparisons between baseline and multiple snapshots of data collected over time, in relation to diurnal variation (Walsh *et al.*, 2006), stressors (Nicholson *et al.*, 1985) and dietary interventions (Phipps *et al.*, 1998). Metabolomics is a platform that uses advanced analytical technology to acquire multivariate datasets from biological samples to comprehensively profile metabolic fluctuations over-time (Beckonert *et al.*, 2007). As a result of metabolomics and biomarker discovery, such work in various fields have advanced the understanding of inborn errors of metabolism relating to disease (Holmes *et al.*, 1997) and the development of pharmaceutical products (Nicholson *et al.*, 2002).

By comparison, exercise science has fallen behind in the expanding fields of metabolomics with a limited number of publications available ever since the first clinical exercise metabolomics study was conducted, to the author's knowledge, in 2005 by a group in Massachusetts, U.S.A (Sabatine *et al.*, 2005). In sports medicine, metabolomics has successfully monitored the progress of exercise interventions, and discriminated between clinical and control populations in relation to myocardial ischemia (Sabatine *et al.*, 2005), ischemia reperfusion in claudication (Coolen *et al.*, 2008), coronary artery disease (Barba *et al.*, 2008), chronic obstructive pulmonary disease (Rodriguez *et al.*, 2012) and diabetes (Kuhl *et al.*, 2008; Huffman *et al.*, 2011; Brugnara *et al.*, 2012). Other studies have demonstrated that discrimination between groups of healthy populations based on metabotypes is possible according to athletic status and/or response to training (Yan *et al.*, 2009; Enea *et al.*, 2010; Kuehnbaum *et al.*, 2014; Santone *et al.*, 2014; Mukherjee *et al.*, 2014), fitness level as determined by VO_{2max} assessments (Chorell *et al.*, 2012; Morris *et al.*,

2013), and long-term participation in physical activity (Kujala *et al.*, 2013). Facilitating research of this nature provides population-specific metabolic information necessary to inform personalised exercise prescription.

Metabolomic efficacy data has also been published on different types of exercise and/or training interventions, including marathon or treadmill running (Lewis *et al.*, 2010; Lehmann *et al.*, 2010), sprint running (Pechlivanis *et al.*, 2010; Pechlivanis *et al.*, 2013; Santone *et al.*, 2014), cycling (Pohjanen *et al.*, 2007; Enea *et al.*, 2010; Chorell *et al.*, 2012; Morris *et al.*, 2013; Kuehnbaum *et al.*, 2014; Mukherjee *et al.*, 2014), football games (Ra *et al.*, 2014), rowing-specific training (Yan *et al.*, 2009) and snowboarding-specific training (Wang *et al.*, 2015). Further work in exercise metabolomics has been published on nutritional ergogenic aids and metabolic recovery following exercise cessation (Chorell *et al.*, 2009; Miccheli *et al.*, 2009; Nieman *et al.*, 2012; Hodgson *et al.*, 2013; Knab *et al.*, 2013). However, the focus of metabolomic studies has been on endurance-based exercise, whereas resistance-based exercise has received limited attention despite its potential to investigate muscular stress from a comprehensive biochemistry perspective. To pursue such an investigation, isokinetic exercise may be an appropriate intervention due to its ability to control multiple exercise parameters of muscle contractions to provide standardised laboratory conditions, as discussed in Section 1.1.

Nevertheless, the targeted analytical approaches commonly employed for metabolic profiling of muscular stress and/or exercise-induced micro-trauma (e.g. enzyme-linked immunoabsorbant assay) are more appropriate for the measurement of the established protein biomarkers (e.g. CK and myoglobin) than metabolomic techniques, despite the limited scope to simultaneously identify multiple untargeted metabolites.

1.3 Metabolomic Analytical Technologies

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the main analytical technologies employed in metabolomic studies for the ability to yield

complex multivariate datasets from biofluid samples (Nicholson *et al.*, 2002). NMR spectroscopy has the advantage of elucidating molecular structures simultaneously on intact biofluid samples with minimal sample preparation required, whereas MS requires prior analyte separation from the matrix by either gas chromatography (GC) following derivatisation or liquid chromatography (LC) (Beckonert *et al.*, 2007). GC-MS and LC-MS have the advantage of high sensitivity with detection limits to the picomolar concentration, whereas the detection limits of NMR spectroscopy are to the micromolar concentration (Lindon and Nicholson, 2008). Thus, the appropriate use of the analytical techniques employed is dependent on the research question or aim of the investigation. For studies that have prior knowledge of a specific class of metabolites that need to be quantified, GC-MS or LC-MS may be appropriate for quantification due to its high sensitivity. For studies with the objective to measure multiple classes of metabolites and/or elucidate molecular structures, ^{1}H NMR spectroscopy may be appropriate due to its ability to simultaneously detect proton resonances assignable to the components of a molecular structure (e.g. aliphatic and aromatic protons). In addition, the non-destructive properties of NMR on biofluid samples allows for further analysis, if necessary, by other techniques at a later stage for cross-validation or quantification purposes.

To carry out a metabolomics study on isokinetic exercise, it is necessary to screen the biofluid samples collected, initially, using a versatile tool with scope to detect and measure multiple classes of metabolites and hence, ^{1}H NMR spectroscopy is the appropriate choice.

1.4 First Principles of ^{1}H NMR Spectroscopy

^{1}H NMR spectroscopy is based on the principle that ^{1}H nuclei possess a non-zero magnetic moment (due to the spin property denoted by the symbol *I*) which can absorb and re-emit electromagnetic energy from applied radiofrequency pulses when positioned in the magnetic field of the instrument (denoted by the symbol B_0) (Evans, 1995). Initially, ^{1}H nuclei flip energy states by lining up either with B_0 (lower energy spin state) or against B_0 (higher

energy spin state) and then radiofrequency pulses are applied to excite the protons. When the magnetic field of B_0 is deactivated, the ^1H nuclei flip back to its original energy state and return to equilibrium (relaxation), and the absorbed electromagnetic energy is re-emitted in the form of resonances which are recorded by the instrument. There are two types of relaxation; spin-lattice/longitudinal relaxation time (T_1 relaxation), time of energy decay as magnetisation reaches equilibrium along B_0 (Figure 1.1A), and spin-spin/transverse relaxation time (T_2 relaxation), time of energy decay across the transverse plane perpendicular to B_0 (Figure 1.1B). Macromolecules (e.g. proteins, lipoproteins and lipids) have short T_2 relaxation times which cause line broadening of the signals, typical in the blood plasma spectrum (Nicholson *et al*., 1995).

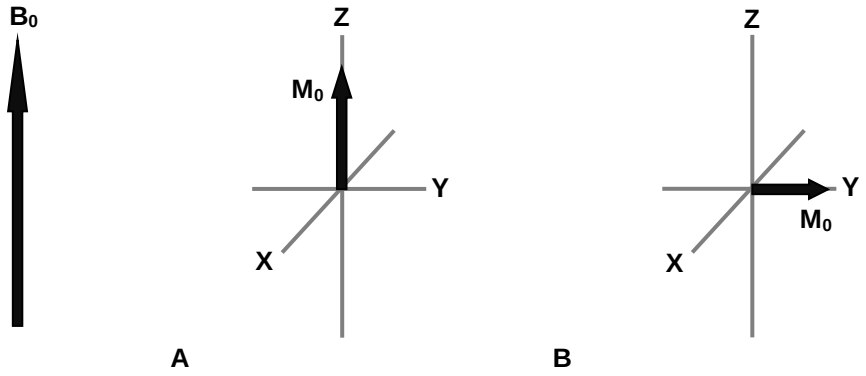


Figure 1.1 ^1H nuclei in a magnetic field (B_0): **(A)** nuclear dipoles (M_0) along B_0 and **(B)** perpendicular to B_0 (diagram adapted from Evans, 1995).

The raw data is acquired in form of free induction decay (FID), as illustrated in Figure 1.2A. Usually, multiple scans are completed and co-added to improve the signal-to-noise ratio; quadrupling the number of scans doubles the signal-to-noise ratio. The FIDs are then Fourier transformed into a spectrum followed by phase and baseline correction for alignment (Figure 1.2B).

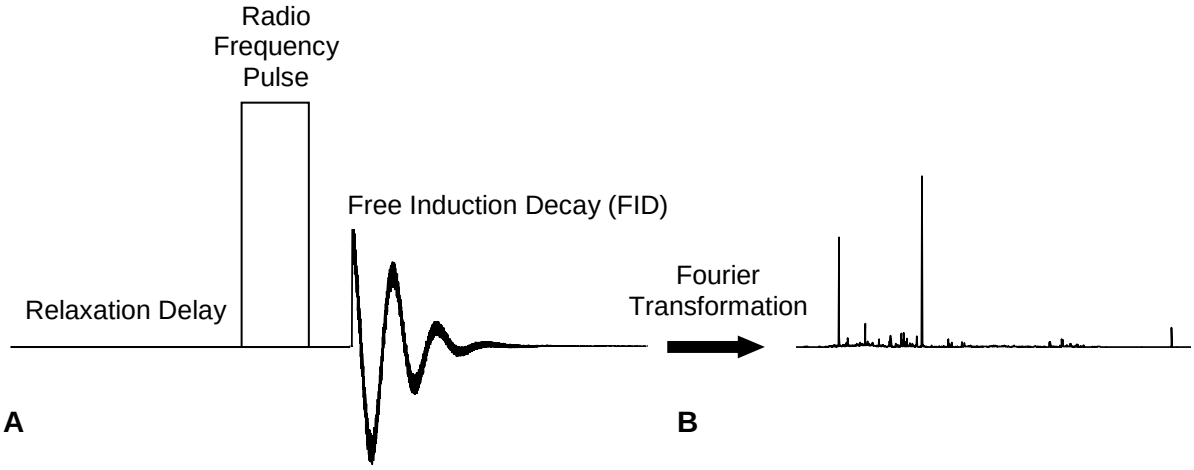


Figure 1.2 (A) Pulse-acquire FID acquisition (diagram adapted from Evans, 1995) and **(B)** Fourier transformation into a spectrum.

The resonance frequency (expressed in Hz or ppm) is dependent on the strength of B_0 and the electronic environment surrounding the ^1H nucleus and thus, indicative of the identity of the molecular component giving rise to the signal; for example, methyl group protons have a lower frequency than methine protons, and aromatic protons have the highest frequencies. Figure 1.3 illustrates the aliphatic (including the sugars and amino acid sub-region) and aromatic regions of the spectrum according to resonance frequencies (ppm).

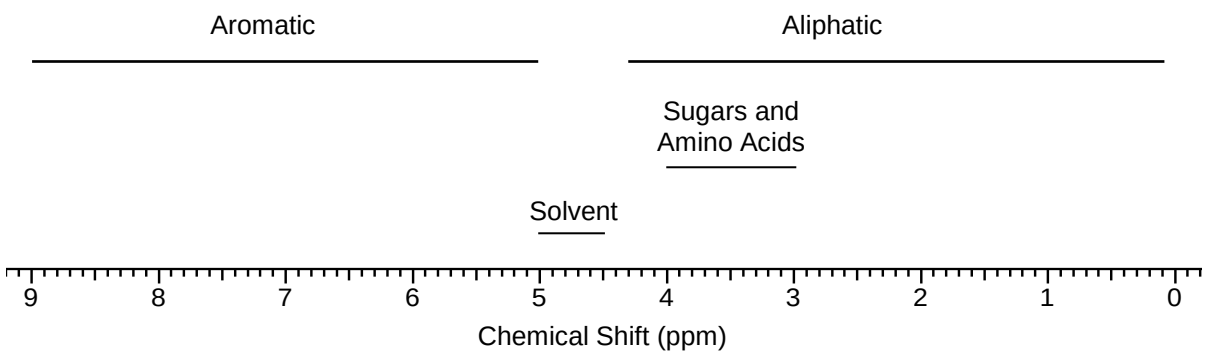


Figure 1.3 ^1H resonance frequency regions of the NMR spectrum.

1.5 ^{1}H NMR Spectroscopy of Biofluid Matrices

^{1}H NMR spectroscopy of biofluids such as saliva (Santone *et al.*, 2014), urine (Mukherjee *et al.*, 2014) and blood plasma (Pechlivanis *et al.*, 2013) has been successfully applied to investigate exercise responses at the molecular level. The metabolic composition of biofluids varies extensively according to its diverse biological roles and interaction with organs or organ systems, and therefore each biofluid possesses a unique ^{1}H NMR spectroscopic signature (Nicholson *et al.*, 2002). The information retrievable from a ^{1}H NMR spectrum of biofluids includes energy absorption frequencies (chemical shifts relative to the internal standard TSP used for aqueous samples) that are characteristic of the proton's chemical environment, spin-spin coupling patterns (multiplicity: Singlet, doublet; triplet; multiplet) from interaction with neighbouring protons and area under the peak of a resonance (Nicholson *et al.*, 2002). The chemical shift values (ppm), denoted by the symbol δ, and multiplicity of the signals are the fundamental features informing resonance assignment to the appropriate molecular components for structural elucidation and identification, whereas the area under the peak is representative of the concentration. Integrating ^{1}H NMR spectra into equal size buckets (e.g. 0.02 or 0.04 ppm) provides numerical values of area under the peaks, which can then be normalised to sum of intensities, TSP or another internal standard (e.g. creatinine signal in urine) to yield semi-quantitative data. The prominent HDO (H_2O and D_2O) solvent signal that arises approximately at δ 4.75 in biofluid spectra can be irradiated by employing water suppression techniques such as presaturation during the delay and mixing time of the NMR experiments using the Nuclear Overhauser Effect Spectroscopy (NOESY) pulse sequence (Nicholson and Wilson, 1989). However, due to the complexity of biofluid matrices, multiple ^{1}H NMR signals overlap in crowded regions of the spectrum which causes difficulty in a number of resonance assignments.

^{1}H NMR spectra of human saliva (Silwood *et al.*, 2002) and urine (Bouatra *et al.*, 2013) both contain many sharp resonances assignable to low-molecular weight metabolites (<1 kDa), although urine is generally more concentrated. The urine matrix is valuable for ^{1}H

NMR spectroscopy studies since it can be collected non-invasively and has features diagnostic of disease (Holmes *et al.*, 1997) and may provide indirect biomolecular markers of the systemic responses to exercise (Enea *et al.*, 2010; Pechlivanis *et al.*, 2010; Mukherjee *et al.*, 2014; Wang *et al.*, 2015). The saliva matrix has also shown potential for metabolomic profiling of exercise as demonstrated in two recent studies on soccer players (Santone *et al.*, 2014; Ra *et al.*, 2014), possibly because the composition of saliva is subject to change depending on the autonomic nervous system which may be perturbed by exercise (Seals and Victor, 1991). The physiology underpinning exercise-induced change in the biomolecular composition of saliva and urine are discussed in further detail in Chapter 3 and Chapter 4, respectively.

Blood plasma contains a high abundance of macromolecules such as proteins, lipoproteins and lipids that give rise to broad resonances, caused by the relatively short proton T_2 relaxation times, with sharp signals of low-weight molecules superimposed on them (Nicholson *et al.*, 1995). The broad resonance signals of macromolecules in blood plasma can be attenuated by employing Carr-Purcell-Meiboom-Gill spin-echo pulse sequences (Nicholson *et al.*, 1995; Daykin *et al.*, 2002), as demonstrated and discussed in Chapter 5. The biomolecular composition of blood plasma/serum is subject to change following exercise due to the transportation of metabolites to and from active skeletal muscle (Viru and Viru, 2001), and thus, plasma/serum provides direct biomarkers of muscular activity during exercise. The metabolic composition of blood serum and its change in response to exercise are discussed in further detail in Chapter 5.

Since ^{1}H NMR spectroscopy of biofluids provides ‘information rich’ multivariate datasets, it is necessary to extract the relevant information related to exercise metabolism using chemometric, pattern-recognition techniques and then use this information to inform the assignment of signals relevant to the investigation.

1.6 Chemometric Analysis of Metabolomic Datasets

Chemometric techniques, including principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA), are commonplace in metabolomics to reduce the complex datasets into two or three dimensional models to provide a visual representation of inherent patterns, if any, in the data (Nicholson *et al.*, 2002). PCA arranges the dataset into uncorrelated vectors termed principal components (PCs) based on the magnitude of data variance, whereby PC1 has a greater magnitude of variance than PC2 and so forth. In a two dimensional model, for example, PC1 and PC2 are plotted orthogonal to each other based on the calculation of latent variables in the form of a scores plot (samples) and loadings plot (weightings of actual variables). Since PCA is an unsupervised technique, whereby no prior information of class is input into the model, it is typically performed to differentiate between normal and idiosyncratic spectra for outlier elucidation, and to describe the biochemical variation and homogeneities in the dataset (Lindon and Nicholson, 2008). PLS-DA is a supervised technique applied to the spectral datasets to discriminate between classes based on a classifier (e.g. stressor) and to elucidate the variable discriminators of the model (Nicholson *et al.*, 2002).

The presentation of the PLS-DA results is similar to PCA, in terms of scores and loadings plots. The interpretation of a PCA model may be approached by visually inspecting the scores plot to determine if any scores fall outside of Hotelling's 95% confidence limits (denoted by an ellipse), to elucidate potential outliers, and observe clustering patterns, if any, indicative of metabolic homogeneities. Figure 1.1 presents an example of a PCA scores plot (without Hotelling's ellipse) of three biofluid ^{1}H NMR datasets (blood serum, urine and saliva) reduced into three dimensions, which shows a separation of scores into three clusters according to the matrix type (class). The cluster divisions in Figure 1.4 are indicative of sample homogeneities in biomolecular composition within class and heterogeneities between classes. In the PCA and PLS-DA loadings plots, the data points (spectral variables)

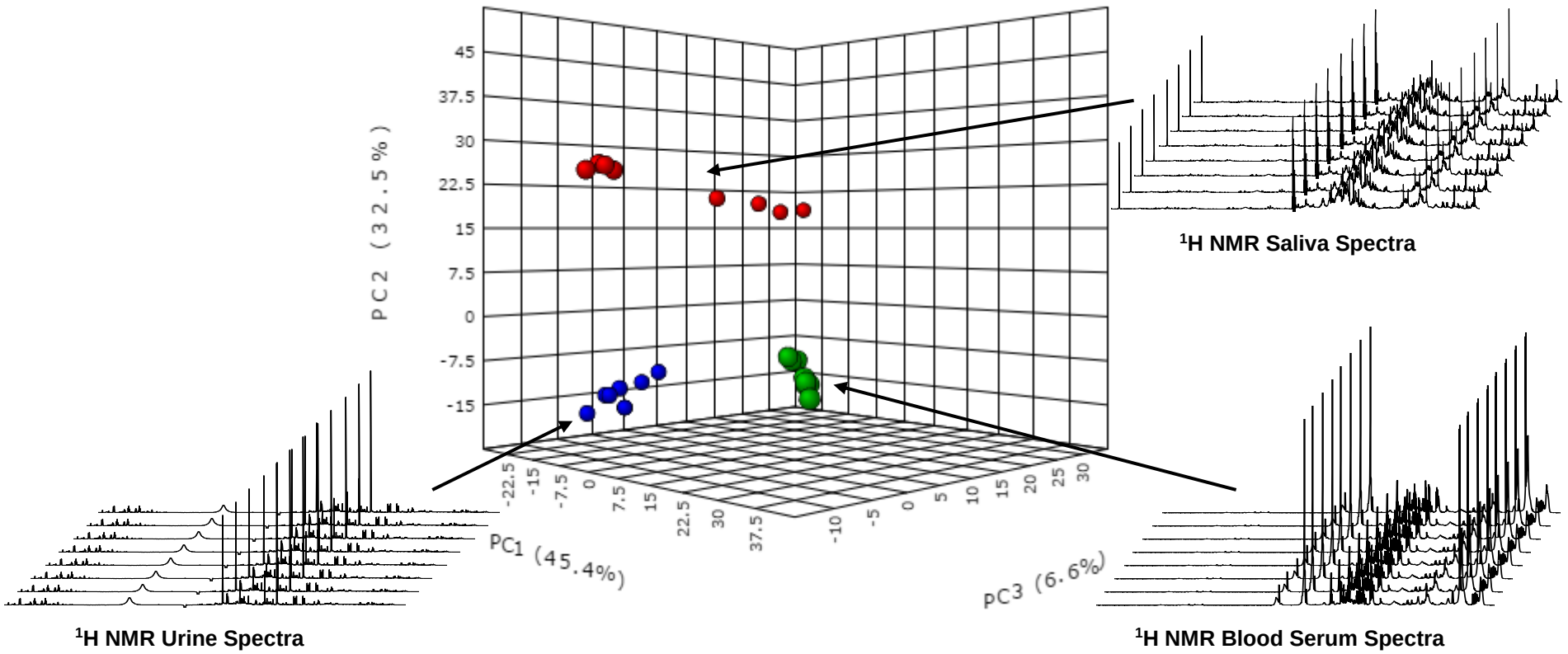


Figure 1.4 Example of a PCA scores plot of three biofluid matrices (red, blue and green circles denote saliva, urine and blood serum, respectively) reduced into three dimensions whereby the largest variance in the dataset is explained by PC1 (45.4%)>PC2 (32.5%)>PC3 (6.6%). A complete class distinction was exhibited between the three biofluid matrices since the scores separated into three clusters according to their class (matrix type). This example was generated from PCA of actual data collected in this study (see Chapter 2 for methods) and presented here to provide a graphical illustration of a PCA model.

which separate from the remaining data points clustered at zero represent the strongest variable weightings on the scores (spectral variables of interest).

A limitation of chemometric data mapping is that inter-individual variation tends to be high in ^{1}H NMR spectral datasets obtained from biofluids (Walsh *et al.*, 2006) and, in some cases, may exceed the effect of the classifier (e.g. the effect of an intervention). Thus, there are extraneous parameters to control, where possible, such as recruiting a homogenous population sample, the implementation of a standardised diet and fluid intake, and collecting biofluids at the same time of day (diurnal cycles) in an attempt to reduce such variation.

1.7 Thesis Aims, Rationale and Original Contribution

This study aims to investigate the effect of concentric isokinetic exercise of the knee extensors and flexors, at high and low intensities of equal workloads at 80%MVC for 10 repetitions and 40%MVC for 20 repetitions, respectively, on blood serum, urine and saliva matrices. The rationale for investigating these two intensities is that muscular stress during exercise is imposed by intensity (e.g. %MVC) over time (e.g. number of repetitions) and thus, standardising these two parameters may yield similar responses, which may have implications for exercise prescription. This study employs a hypothesis-free approach to mathematically determine, by chemometrics, which untargeted metabolites measured by ^{1}H NMR spectroscopy may be associated with muscular exercise of this nature.

To the author's knowledge, there are currently no metabolomics data published on the acute response to resistance-based exercise. The acquisition of such data may further elucidate the behaviour of low-molecular weight biomolecules during concentric loading, which may be indicative of the systemic response to muscular stress. This research may have implications for prospective exercise studies including sourcing information from non-invasive matrices (urine and saliva), metabolic health assessment, and metabotype discrimination between subgroups and individuals to inform exercise prescription for personalised healthcare.

CHAPTER 2: METHODS

2.1 Research Participants

This research study was approved by the University of East London's Research Ethics Committee (UREC) on 8th November 2012 (Appendix 2.1). All data obtained from participants were anonymised to protect identity and stored on a password protected computer in accordance with University of East London's policy.

Non-smoking, healthy men (free from injury, illness and medication) of autonomous mind, aged 18-40 years, from the University of East London (UEL) were invited to participate in this research. The rationale for excluding women was that marked differences according to gender were reported in the NMR profiles of metabolites in saliva (Takeda *et al.*, 2009), urine (Slupsky *et al.*, 2007) and blood serum (Psychogios *et al.*, 2011). The rationale for excluding participants above 40 years of age was that marked differences in NMR profiles of metabolites in the urine have been reported according to age, categorised by above and below 40 years of age (Slupsky *et al.*, 2007).

Participant recruitment was advertised internally via bulletins on the UEL website and open invitations to the author's colleagues and acquaintances. Sixteen volunteers expressed their interest in taking part and then received an information letter outlining the nature of this study and any potential risks/burdens involved in participation (Appendix 2.2). The volunteers were permitted a period of two weeks from receiving the information letter to make their decision about participation. All sixteen volunteers agreed to take part and therefore provided informed signed consent to participate including permission to publish any anonymised data obtained (Appendix 2.3). No payment was offered for participation and all participants were free to withdraw from the study at any time without disadvantage to themselves.

Prior to participation, each volunteer completed a Physical Activity Readiness Questionnaire (PARQ) and health screening form taken from the American College of Sports Medicine (ACSM) (Appendix 2.4), to ensure that they fulfilled the aforementioned inclusion criteria for this study (free from injury and illness, and not on medication at the time of participation). The final inclusion criterion was that the volunteers were not enrolled as participants in another study during the present study. Fifteen of the sixteen volunteers fulfilled the inclusion criteria and were recruited to participate in the study. One participant withdrew from the study due to work commitments prior to participation, which reduced the population sample size to fourteen individuals. The rationale for not using a power calculation to estimate the population sample size was that such calculations are based on univariate datasets, whereas metabolomics generates multivariate datasets and thus, deemed inappropriate in the present study. The minimal population sample size was set to twelve individuals since this matched the smallest sample size utilised in the published literature on exercise metabolomics at the time of which this study was designed (Pechlivanis *et al*., 2010). However, fourteen individuals were available to take part within the time frame of this study and were therefore recruited.

Each participant was instructed to refrain from strenuous exercise and the consumption of alcohol and caffeine for 48 hours prior to participation, in an attempt to standardise the conditions, and to keep hydrated. Participants were also instructed to bring comfortable clothing that they felt was appropriate for exercise on the days of participation. The participants were provided with a pre-exercise testing, standardised eating plan taken from Gore (2000) to reduce the influence of dietary differences on biofluid matrices (Appendix 2.5). This eating plan was originally designed to be followed for three days prior to exercise testing in athletes. Standardising nutritional intake is presented with the challenges of offering food choices that are representative of a normal diet and determining the length of time necessary to implement the plan without causing an inconvenience for the participants. Walsh *et al.* (2006) and Winnike *et al.* (2009) reported that implementing a standardised diet

for a 24 hour period was sufficient to normalise NMR metabolite profiles in urine. Thus, the eating plan presented in Appendix 2.5 was adapted from the original three-day standardised diet to 24 hrs prior to biofluid collection.

2.2 Study Design

The fourteen participants attended the muscle function testing laboratory at the University of East London, Stratford Campus, on three separate occasions; each visit was 3-7 days apart. Since diurnal variation has a strong influence on NMR profiles of metabolites in saliva, urine and blood (Walsh *et al.*, 2006), all three visits took place at the same time of day (specifically at 9 am) and lasted up to 2.5 hours. After consuming the standardised breakfast, participants were instructed to fast until the end of the session to avoid nutritional modulated change, but were permitted to drink water for their comfort.

The first visit was arranged as a preliminary session to measure participant descriptive characteristics (age, height and body mass) and force output (N) of the knee extensor and flexor muscles during maximal voluntary isokinetic contractions (MVC), and for familiarisation of isokinetic exercise. The protocol for the determination of MVC force output is described in Section 2.3.3. The results from this preliminary session are presented in Table 2.1 and the force output data was used to calculate 80% and 40%MVC for the subsequent visits.

The second visit was arranged to complete one of two exercise interventions in a block randomised order; participants 01, 03, 05, 07, 09, 11 and 13 completed an intervention at 80%MVC, whereas participants 02, 04, 06, 08, 10, 12 and 14 completed an intervention at 40%MVC. Thus, on the third visit, the sessions were reversed to ensure that all participants completed both interventions. The interventions at 80%MVC and 40%MVC are referred to as high intensity exercise (HIE) and low intensity exercise (LIE), respectively. The protocols for the HIE and LIE interventions are described in Section 2.3.4.

Table 2.1 Participant descriptive characteristics and force output data

	Age	Height	Mass	BMI	Left Leg MVC		Right Leg MVC		Exercise History	
Participant	**(Years)**	**(m)**	**(kg)**	**(kg/m$^{2)}$**	**Ext (N)**	**Flex (N)**	**Ext (N)**	**Flex (N)**	**Classification**	**Frequency**
1	28	1.60	70	27.34	450.4	269.8	420.8	259.2	Mod. Active	1-3 per week
2	24	1.68	75	26.57	505.2	217.6	586.4	264.4	Mod. Active	1-3 per week
3	25	1.80	86	26.54	547.8	328.8	552.0	331.0	Athletic	≥6 per week
4	18	1.83	80	23.89	420.2	371.8	363.6	310.4	Athletic	≥6 per week
5	26	1.73	82	27.40	476.6	186.2	356.2	206.6	Sedentary	0 per week
6	19	1.76	70	22.60	498.4	279.8	667.0	358.6	Mod. Active	1-3 per week
7	23	1.79	89	27.78	617.8	296.0	585.8	333.0	Active	4-5 per week
8	30	1.82	82	24.76	430.4	184.6	346.4	163.4	Athletic	≥6 per week
9	39	1.80	78	24.07	559.4	249.6	614.2	292.2	Athletic	≥6 per week
10	33	1.79	95	29.65	590.8	301.6	573.4	320.0	Active	4-5 per week
11	25	1.88	75	21.22	671.0	285.8	672.4	279.8	Mod. Active	1-3 per week
12	31	1.74	80	26.42	399.2	158.2	390.0	171.4	Active	4-5 per week
13	22	1.66	70	25.40	648.0	346.4	657.8	416.0	Active	4-5 per week
14	19	1.73	76	25.39	496.8	183.4	497.8	196.6	Active	4-5 per week
Mean	26	1.76	79	26.10	522.3	261.4	520.3	278.8		
SD	6	0.07	7	2.01	83.0	64.4	117.7	71.3		

The exercise classification was based categorically on the frequency of exercise per week according to the ACSM PAR-Q self-assessment form.

To measure the metabolic responses to both HIE and LIE, samples of venous blood, urine and saliva were collected on ice from each participant pre- and post-exercise, stored at -80°C (unless otherwise stated), then thawed and prepared for NMR spectroscopic analysis, as described in Section 2.4. Methods of data acquisition, statistical analysis and metabolomic interpretation are described in Sections 2.5, 2.6 and 2.7, respectively.

2.3 Isokinetic Dynamometry

2.3.1 Warm-up Exercise

A 10 min warm-up exercise was employed, prior to isokinetic exercise, at 50 W on an electronically-braked bicycle ergometer (Lode B.V. Medical Technology; Groningen, Netherlands), with the seat adjusted to hip-height to account for lower-limb length and handlebars within reach. All adjustments were recorded for subsequent sessions.

2.3.2 Instrument Setup

All isokinetic exercise protocols were carried out on a KinCom isokinetic dynamometry chair (KinCom 500H; Chattecx Corp., Inc., Hixson, TN) and the instrument was set up according to the manufacturer's instructions. Due to the instrument design, exercise was performed separately on both legs.

The length of the seat on the dynamometry chair was adjusted so that the participant's back was supported and the upper-legs rested comfortably on the seat. Each participant was secured to the dynamometry chair via a waist strap to stabilise the pelvis and an upper-leg strap to help isolate the working muscle groups. The axis of the lever-arm of the dynamometer was mechanically aligned both horizontally and vertically with the axis of the working knee (space between the head of fibula and lateral epicondyle of the femur) to ensure that smooth movements were performed comfortably. The cuff of the lever-arm was strapped to the working leg just above the ankle. All adjustments were recorded for subsequent sessions.

The effect of gravity on the strain gauge, caused by the mass of the limb, was compensated by measuring and recording the mass of the working leg while at full extension of the knee. The range of motion was set from 90° of knee flexion to full extension with the application of safety stop mechanisms to prevent the lever-arm from over turning. The angular velocity was set to 60°.s^{-1} and the mode of contraction was set to concentric. A visual display on the monitor of the dynamometer system provided real-time graphical feedback of force production (N).

2.3.3 Determination of MVC Force Output

With verbal encouragement, each participant pushed upward on the cuff with maximal effort until full knee extension was reached and then pushed downward with maximal effort until return to start position (90° of knee flexion) for 5 continuous repetitions, to obtain a mean force output.

2.3.4 HIE and LIE Interventions of Standardised Workloads

The parameters (%MVC, number of repetitions and sets, and duration of rest intervals) were set according to the ASCM guidelines (ACSM, 2002). For the HIE intervention, force markers were set to 80%MVC for each individual and visually displayed on the monitor as a guide of how much effort to apply to the exercise. Ten repetitions of knee extension and flexion to 90° were completed for 4 sets, with 1 min rest intervals.

For the LIE intervention, force markers were set to 40%MVC and displayed on the monitor as a guide. Twenty repetitions of knee extension and flexion were completed for 4 sets with 1 min rest intervals. The work completed was standardised (repetitions x %MVC) for both sessions.

2.4 Biofluid Collection, Storage and Preparation for ^{1}H NMR Spectroscopy

2.4.1 Time Points of Collection

Samples of saliva, urine and venous blood were collected from the 14 participants and frozen on site from 9.00 am on arrival, prior to exercise, as described below and illustrated in Figure 2.1. The HIE and LIE sessions started from 9.30 am and finished at approximately 10.10 am. Further samples of saliva, urine and venous blood were collected immediately after exercise and frozen at -80°C (unless otherwise stated). A final venous blood sample was collected one hour into the recovery phase. The exact times of biofluid collection and exercise participation were recorded for each individual for accuracy.

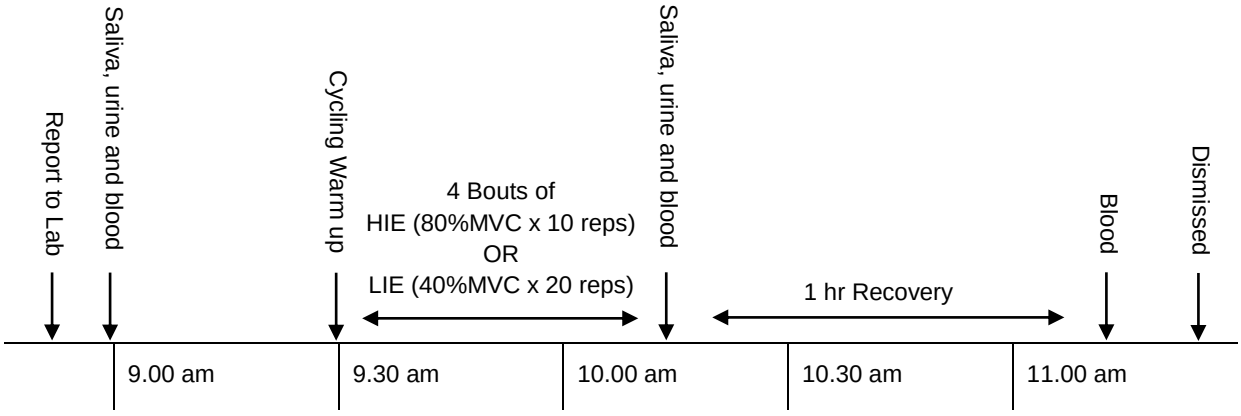


Figure 2.1 Timeline of biofluid (saliva, urine and blood) collection in this isokinetic exercise study.

2.4.2 Saliva

Whole saliva samples were collected on ice from the fourteen participants using salivettes (Salimetrics, UK) to promote hygienic, unstimulated collection with a low risk of spillage. The effect of different types of saliva sampling techniques on NMR profiles of metabolites and justification for the use of salivettes in the present study are discussed in

Chapter 3. Each participant rinsed their mouth with drinking water in an attempt to remove any food debris and then placed the salivette onto the tongue and closed their mouth for 2 min, without chewing to avoid the effect of mechanical stimulation. The salivette was then placed into a double chambered container and centrifuged at 1,500 x *g* for 15 min at 4°C (MSE Mistral 3000i, MSE; UK) to retrieve the absorbed saliva according to the manufacturer's instructions. Various quantities of saliva ranging from 0.5-2.0 mL were extracted from each individual salivette and were frozen immediately at -80°C.

For analysis, the 56 frozen saliva samples were thawed on ice and centrifuged at 3,500 x *g* for 20 min to remove any cellular debris (The Laboratory Standard Microcentrifuge 5424, Eppendorf; UK). All reagents utilised in this study were purchased from Sigma-Aldrich. In accordance with Silwood *et al.* (2002), a 5 mM solution of the internal standard 3-trimethylsilyl(-2,2,3,3-$^{2}H_4$) propionate (TSP) in deuterium oxide (D_2O) was prepared. The TSP provided a chemical shift reference signal at 0.00 ppm and the D_2O provided a frequency field lock for B_0 magnetic field shimming. This solution was modified by adding a pH 7.4 phosphate buffer (0.2 M Na_2HPO_4 and 0.04 M NaH_2PO_4), to control for saliva pH differences between samples, and 3 mM sodium azide (NaN_3) to prevent bacterial preponderance and metabolism. 100 µL of each sample supernatant was added to 400 µL of the prepared solution. The prepared saliva samples (500 µL) were centrifuged at 12,000 x *g* for 15 min at 4°C and then transferred into 5 mm NMR tubes for analysis.

2.4.3 Urine

Each participant was provided with a 220 mL sterile, leak-proof, specimen container sealed with a screw on lid and instructed to provide a spot void urine sample collected mid-stream (using campus facilities), which was placed on ice for transportation to the laboratory. The sample volumes provided by each participant in the sterile containers ranged between 50-200 mL and were frozen immediately at -20°C.

For analysis, the 56 frozen urine samples were thawed at room temperature. 2 mL of each urine sample was transferred into eppendorfs and centrifuged at 3,500 x *g* for 15 min to remove any cellular debris (The Laboratory Standard Microcentrifuge 5424, Eppendorf; UK) and prepared according to Beckonert *et al.* (2007). 400 µL of urine supernatant was added to 200 µL of a pH 7.4 phosphate buffer (0.2 M Na_2HPO_4 and 0.04 M NaH_2PO_4) containing 1 mM TSP, 10% D_2O and 3 mM NaN_3. The prepared urine samples were centrifuged at 12,000 x *g* for 15 min at 4°C and then 550 µL of each sample was transferred into 5 mm NMR tubes for analysis.

2.4.4 Venous Whole Blood and Serum Extraction

Venous whole blood samples were collected in compliance with the World Health Organisation (WHO) guidelines on drawing blood: best practices in phlebotomy (WHO, 2010). 5 mL of venous blood was collected from a forearm vein via peripheral venepuncture using a 21G butterfly needle (BD Safety-Lock Blood Collection Set, 21G, BD; U.K.) from the fourteen participants, whilst in a seated position. Prior to inserting the needle, a tourniquet was fastened over the upper arm, approximately 2 cm above the elbow, to raise the veins of the forearm towards the surface. The area of skin at the site targeted for venepuncture was cleaned with an alcohol wipe and left for 30 s to dry. The needle was inserted through the skin into the vein, until blood flash-back was seen in the catheter, which indicated that the needle was in the correct position. A 5 mL serum vacutainer (BD Vacutainer SST Advance Tube, 5 mL, BD; U.K.) was attached to the end of the catheter, via the plastic holder and vacutainer needle, until completely filled with whole blood. The tourniquet was removed during the bleeding. The blood-filled vacutainer was detached from the catheter and placed on ice. The needle was removed and a sterile dressing was placed upon the puncture where pressure was applied immediately until bleeding had stopped. A plaster was used to dress the puncture. The aseptic non-touch technique was applied throughout.

The vacutainer was inverted 5 times to homogenise the sample and kept on ice to coagulate for 1 hr to remove the clotting factors, according to BD manufacturer's

instructions. To obtain serum, the vacutainer was centrifuged at 2500 x *g* for 15 min at 4˚C (MSE Mistral 3000i, MSE; UK), according to Beckonert *et al.* (2007). 2 mL of serum supernatant was transferred from the whole blood into eppendorfs and stored at -80°C.

For analysis, the 84 frozen blood serum samples were thawed at room temperature. 1 mL of blood serum was transferred into eppendorfs and centrifuged at 3500 rpm for 15 min to remove any cellular debris (The Laboratory Standard Microcentrifuge 5424, Eppendorf; UK) and prepared according to Beckonert *et al.* (2007). 200 µL of the serum supernatant was added to 400 µL of a pH 7.4 phosphate buffer (0.2 M Na_2HPO_4 and 0.04 M NaH_2PO_4) containing 1 mM TSP, 10% D_2O and 3 mM NaN_3. The prepared blood serum samples were centrifuged at 12,000 x *g* for 15 min at 4°C and then 550 µL of each sample was transferred into 5 mm NMR tubes for analysis.

2.5 ^{1}H NMR Spectroscopy of Biofluid Samples

2.5.1 Data Acquisition

Spectral acquisition parameters for biofluids were set according to Beckonert *et al.* (2007). ^{1}H NMR spectra were acquired from all biofluid samples on a Bruker Avance III 600 spectrometer (Karlsruhe, Germany) at an operating frequency of 600.13 MHz. Saliva and urine spectra were acquired at the School of Biological and Chemical Sciences, Queen Mary University London, using a 5 mm TXI probe at a temperature of 303 K. In an attempt to overcome the interfering HDO signal of saliva and urine spectra, all experiments were performed using the one-dimensional Nuclear Overhauser Effect Spectroscopy (1D NOESY) pulse sequence with presaturation during the relaxation delay and mixing time for water suppression. The 1D NOESY pulse sequence is as follows: -RD-90°-*t*-90°-t_m-90°-ACQ (RD = relaxation delay; 90° = 90° radiofrequency pulse; *t* = short delay of 3 µs; t_m = mixing time; ACQ = acquire FID). 256 FIDs were collected into 32,768 time domain data points using a spectral width of 8,389 Hz, a relaxation delay of 2.0 s, a mixing time of 0.1 s and an acquisition time of 3.91 s.

Blood serum spectra were acquired at The National Phenome Centre, Imperial College London, using a 5 mm PABBI 1H/D-BB Z-GRD probe at a temperature 309 K. An initial experiment was carried out on all blood serum samples using the 1D NOESY pulse sequence with presaturation as detailed above. In an attempt to reduce the effect of blood serum macromolecules on the line broadening of resonance signals, a spin-echo experiment was performed using a 1D Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with presaturation during T_2 relaxation. This spin-echo experiment permits spectral editing via the attenuation of signals from macromolecules (Nicholson *et al.*, 1995; Daykin *et al.*, 2002). The CPMG pulse sequence is as follows: -RD-90°-(t-180°- t)$_n$-ACQ (t = spin-echo delay; 90° or 180° = 90° or 180° radiofrequency pulse, respectively; n = number of loops, in this experiment n = 80). Since blood serum is more concentrated than saliva and urine, less scans were needed to acquire adequate serum spectra. Thus, 32 FIDs were collected into 36,864 time domain data points using a spectral width of 12, 019 Hz, a relaxation delay of 2.0 s, a spin-echo delay of 400 µs and an acquisition time of 3.07 s.

To investigate potential interference from salivette exposure on ^{1}H NMR signals, a routine pulse-acquire experiment was performed using the Bruker standard 1D ZGPR pulse sequence on a pure water sample exposed to a salivette and treated according to the saliva preparation methods. Data was acquired on a Bruker Avance III 400 spectrometer (Karlsruhe, Germany) at an operating frequency of 400.13 MHz, equipped with a BBI at a temperature of 295 K, at the Leicester School of Pharmacy, De Montfort University, Leicester. 8 and 128 FIDs were collected into 32,768 time domain data points using a spectral width of 8,277 Hz, a relaxation delay of 2.0 s, a mixing time of 0.1 s and an acquisition time of 3.96 s.

2.5.2 Data Processing and Integration

All FIDs were processed using ACD Labs NMR processor freeware (available for download at www.acdlabs.com/resources/freeware/nmr_proc/). According to Beckonert *et al.* (2007), a line broadening factor between 0.3-1.0 Hz is necessary to improve the signal-to-

noise ratio, to aid the differentiation between true ^{1}H NMR signals and baseline noise. However, the application of exponential line broadening to improve the signal-to-noise reduces spectral resolution creating difficulty in visual determination of signal multiplicity. Thus, 0.5 Hz exponential line broadening was applied to the FIDs to comprise between an improvement in the signal-to-noise ratio of each spectrum and minimal loss of spectral resolution. Each FID was Fourier transformed into a spectrum and manually phase and baseline corrected, and the chemical shift (δ) was referenced to TSP at 0 ppm to adjust any offsets. Dark regions were set to exclude TSP (δ -2.25-0.70), the HDO signal (δ 4.50-5.50 in saliva and urine, and 4.50-5.00 in blood serum) and empty regions (δ 9.00-11.78).

The spectra (excluding the dark regions) were integrated into spectral buckets: a 0.04 ppm bucket size was employed for saliva and urine, since it provides a balance between resolution and covering a typical width of a ^{1}H NMR signal, taking into account spin-spin coupling (Craig *et al.*, 2006); a 0.02 ppm bucket size was employed for blood serum since a greater resolution is required to account for broad signals of macromolecules overlapping with metabolite signals. Each bucket (integral) provided a numerical value of area under the peak (or partial peak) contained within the integral, which provided semi-quantitative data representative of metabolite concentrations that was then used for statistical analysis.

2.6 Statistical Analysis of Biofluid ^{1}H NMR Spectral Datasets

2.6.1 Multivariate Statistical Analysis by Chemometrics

Data normalisation and chemometric analysis were carried out using the freely accessible, online platform Metaboanalyst Version 3.0 (available at http://www.metaboanalyst.ca/MetaboAnalyst/). Data obtained from each biofluid type and exercise intervention (HIE and LIE) were subjected to separate chemometric analysis. Each spectrum was normalised to the sum of intensities (total integrals) of the dataset, so that each data point represents a fraction of the total spectral integral, to account for differences

in samples volumes obtained for saliva and urine, and inter-individual differences in biofluid concentrations (Craig *et al.*, 2006). Urine spectral data were also normalised to the creatinine signal at δ 3.05 ppm according to Pechlivanis *et al.* (2010), to account for differences in glomerular filtration rates as discussed in Chapter 4. Pareto scaling (data mean centred and divided by the square root of standard deviations of each variable) was applied to each dataset for column-wise normalisation so that the weighting represents the covariance of the variables (Craig *et al.*, 2006).

Principal Component Analysis (PCA) was performed separately on all spectral datasets to investigate the effect of HIE and LIE on biofluid metabolites without the influence of a predetermined classifier (unsupervised) and to detect and manage potential outlying spectra, since outliers can mathematically skew data models and usually fail to provide information coherent with representative scores. PCA model refinement, if required, is context dependent and thus, the results from initial PCA informed the appropriate refinement approaches to employ thereafter to obtain the final data models, as presented and discussed in the results chapters.

PLS-DA was performed separately on all spectral datasets to discriminate between classes with exercise as the classifier (supervised) and to elucidate spectral integrals associated with the classifier to inform resonance assignment to metabolites relevant to this investigation.

2.6.2 Univariate Statistical Analysis

Since the ^{1}H NMR integrals of urinary creatinine (δ 3.05 and 4.06), urea (δ 5.80) and lactate (δ 1.33 and 4.13) dominated the PCA loadings plots for both HIE and LIE as reported in Chapter 4, univariate statistical analysis was carried out on these integrals to confirm if there was a statistically significant difference pre- and post-exercise in this study to inform PCA refinement. Univariate statistical analysis was carried out using SPSS 20 (IBM, Armonk, NY, USA). The Shapiro-Wilk test of normality distribution was employed to

determine parametric data assumptions (≥0.05) and the results are presented in Appendix 2.6. Either the Paired Sample *t*-Test (Appendix 2.7) or Wilcoxon Signed Rank Test (Appendix 2.8) was employed as appropriate to determine if there is a statistical significant difference (≤ 0.05) between pre- and post-HIE and LIE values of urinary creatinine, urea and lactate. The Spearman's rho correlation coefficient was applied to investigate associations between the urinary variables creatinine and urea, and creatinine and lactate.

2.7 Metabolomic Interpretation

Spectral resonances elucidated by the chemometric models as being of potential relevance to this exercise study were manually assigned to the biomolecular components which gave rise to the signals, based on chemical shift values (δ) and signal multiplicity according to the available literature, as reported in the individual results chapters, and the online Human Metabolome Database (available at www.HMDB.ca).

The potential origins and functions of the identified metabolites were confirmed via the use of the online Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping database (available at http://www.genome.jp/kegg/pathway.html) and appropriately cross-referenced against the available literature on human and bacterial biochemical pathways, as appropriately cited in the individual results chapters.

CHAPTER 3: ^{1}H NMR SPECTROSCOPY RESULTS OF SALIVA SAMPLES AND CHEMOMETRIC MODELLING OF HIE AND LIE

3.1 Background

Muscular exercise induces a sympathetic-adrenal response via the autonomic nervous system (Seals and Victor, 1991). The autonomic nervous system modulates the activation of acinar cells of the salivary glands (parotid, submandibular and sublingual), amongst other functions, and therefore influences the salivary flow rate and the biomolecular composition of saliva (Kaufman and Lamster, 2002). Briefly, the parasympathetic nervous system induces vasodilation to promote an increased blood supply to the salivary glands, whereas the sympathetic nervous system induces the opposite effect, yet both neurological pathways can stimulate the salivary glands. The parasympathetic pathway stimulates the parotid and submandibular glands, specifically, whereas the sympathetic pathway stimulates the sublingual and other minor glands within the oral cavity (Baum, 1987). Gland-specific saliva has been reported to differ in biomolecular composition (Takeda *et al.*, 2009) and thus, saliva analysis may be informative of gland-specific physiology and the neurological pathways involved in gland stimulation (indirectly). Whole saliva also contains blood plasma constituents, transferred from the capillaries facilitated by intracellular and extracellular pathways via either active transport or passive diffusion (Kaufman and Lamster, 2002), which may be indirectly informative of systemic metabolic processes.

To that effect, numerous studies have measured the concentration of targeted biomolecules in saliva, such as lactate, to serve as indirect markers of exercise-induced systemic perturbations; however, mixed results were reported in the literature (Mendez *et al.*, 1976; Segura *et al.*, 1996; Bocanegra *et al.*, 2012). Although marked increases were documented in salivary lactate concentration from approximately 0.25 to 3.00 or 4.25 mM depending on the exercise (treadmill running, cycling or swimming), Mendez *et al.* (1976)

reported no correlation between saliva and blood lactate levels (an established blood biomarker of exercise intensity and tolerance), whereas Segura *et al.* (1996) and Bocanegra *et al.* (2012) reported a strong correlation ($r > 0.90$). Other salivary biomolecules that have served as markers of exercise stress include the protein chromogranin A (Bocanegra *et al.*, 2012), the digestive enzyme α-amylase (de Oliveira *et al.*, 2010) and the stress hormone cortisol (Neary *et al.*, 2002), all of which have been reported to have a strong correlation with blood lactate levels, indicative of anaerobic metabolism during exercise. Further work by Mulder *et al.* (2009) proposed salivary dehydroepiandrosterone (endogenous steroid) and lactoferrin (globular glycoprotein) as potential biomarkers of imposed muscular stress since marked differences were reported after 4 bouts of squatting exercise for 10 repetitions. However, these potential salivary biomarkers were not correlated to blood dehydroepiandrosterone and lactoferrin as a benchmark in the study. Other studies have investigated the effect of long-term (6-12 months) training on mucosal immunity via measuring salivary immunoglobin A by enzyme-linked immunoabsorbant assay and found that moderate training had a positive effect on the elderly, aged around 65 (Akimoto *et al.*, 2003), and strenuous training had a negative effect on elite swimmers (Gleeson *et al.*, 1995). These studies clearly demonstrate the value of saliva as a non-invasive source of metabolic and immunological information for targeted analysis in sports medicine.

Recently, the potential of the saliva matrix as a source of biochemical information in exercise metabolomics has been demonstrated by two studies (Santone *et al.*, 2014; Ra *et al.*, 2014). Santone *et al.* (2014) investigated the effect of a Yo-Yo exercise test (indirect measurement of aerobic fitness employed by coaches to athletes) on salivary metabolites, as measured by ^{1}H NMR spectroscopy. The findings elucidated biomarkers of anaerobic glycolysis (lactate), glucose-alanine cycle (glucose and alanine), protein degradation (urea) and transamination/amino acid metabolism (glutamate and leucine), fatty acid metabolism (glycerol), tricarboxylic acid cycle intermediates (citrate), and acetate (bacterial metabolism). Since the Yo-Yo test is related to aerobic fitness based on interval running performance,

these biomolecules may serve as potential markers of sports performance. Data obtained from the Yo-Yo test (heart rate ($b.min^{-1}$) and total distance ran (m)) were inserted into the ^{1}H NMR integral dataset for PCA, which discriminated between 'good', 'normal' and 'bad' performers. Since PCA employs algorithms to determine the maximal variance in the dataset, it is unlikely that the mixed dataset (integrated NMR-metabolomic data combined with physiological variables in the bucketed spreadsheet) exclusively represented biochemical variation based on performance and possibly biased the metabolomic dataset with extraneous variables. Possibly, it would have been more appropriate to use the pre-determined subgroups (good, normal and bad performers) based on the physiological data, in isolation, to define the classifier and then employ PLS-DA of the NMR-metabolomic dataset to discriminate between the classes (subgroups).

Ra *et al.* (2014) investigated the effect of fatigue induced by three consecutive 90 minute soccer games on salivary metabolites, as measured by GC-MS, together with the collection of data on heart rate, body mass and Profile of Mood States (psychological test) to be indicative of fatigue. Baseline saliva samples and fatigue data were collected at rest one month prior to the games to avoid pre-game stress and post-game samples were collected at rest one day after the final game. Since the analysis of biological samples provides a snapshot of data informative of the state of an individual at the time of collection, potential change in health, stress or physiological state of the participants within the month between sampling and the games was unaccounted for in the study. The findings of the study revealed a significant increase in a number of salivary biomolecules and fatigue markers (increased heart rate, decreased body mass and, as a result of the Profile of Mood States, increased fatigue and vigour scores) following the games. Specifically, an increase was reported in the concentrations of salivary markers of protein degradation and amino acid metabolism (Leucine, isoleucine, valine, alanine, tryptophan, tyrosine and phenylalanine), and glycogenolysis (glucose 6-phosphate and glucose 1-phosphate). Thus, these salivary biomolecules may be associated with fatigue induced by multiple soccer games and

therefore potentially relevant to sports medicine to inform the extent of fatigue during the competitive season.

Although the saliva matrix has shown promise for exercise metabolomic studies, there are multiple limitations and challenges to overcome. Whole saliva is a host for bacterial metabolites (*n*-butyrate, lactate, acetate and formate), gingival crevicular fluid, expectorated bronchial and nasal secretions, derivatives from oral abrasions, viruses and fungi, cellular components and food debris (Kaufman and Lamster, 2002). Such extraneous variables may extensively affect the concentrations of low molecular weight metabolites in saliva, in particular, bacterial preponderance and metabolism. Since whole saliva is exposed to the oral environment, the use of oral hygiene products may influence the bacterial content of saliva and thus, influence bacterial metabolites present in saliva (Grootveld *et al.*, 2009; Lemanska *et al.*, 2012). The application of sodium azide to all types of biofluid samples to prevent further bacterial metabolism prior to analysis is a routine procedure in ^{1}H NMR spectroscopy-based studies. However, this method does not account for bacterial preponderance and metabolism prior to biofluid collection. The metabolic composition of saliva may also be affected by diurnal cycles (Walsh *et al.*, 2006; Bertram *et al.*, 2009), gender differences and smoking (Takeda *et al.*, 2009). In the present study, whole saliva was collected at 9 am from non-smoking, healthy men in an attempt to avoid such variation.

Saliva composition has also been reported to be influenced by the method of collection employed. Salivette collection has been widely used in sport and exercise science to measure targeted biomarkers and has recently been employed in metabolomics (NMR- or GC-MS-based) in an exercise setting (Santone *et al.*, 2014; Ra *et al.*, 2014) and in a non-exercise setting (Walsh *et al.*, 2006; Bertram *et al.*, 2009). Concentrations of salivary cortisol and IgA have been reported to be lower in samples collected by salivettes compared to passive drool (Strazdins *et al.*, 2005; Takagi *et al.*, 2013), and Walsh *et al.* (2006) and Bertram *et al.* (2009) both suggested that salivette collection may cause ^{1}H NMR interference in the spectral region of δ 3.00-4.00 however, no spectrum showing salivette

background noise was presented in these studies. Nevertheless, salivettes promote hygienic sampling with a low risk of spillage compared to passive drool collection and are convenient for mass sampling. Takeda *et al.* (2009) investigated the effect of taste stimulation (the application of a citric acid solution to the tongue) on ^{1}H NMR metabolite profiles of whole saliva using the passive drool collection method and found that saliva was more dilute following gland stimulation compared to unstimulated collection.

Salivary metabolomics (NMR- or GC-MS-based) in sport and exercise science is in its infancy and recent studies have focused on profiling metabolic perturbations induced by interval running (Santone *et al.*, 2014) and soccer (Ra *et al.*, 2014), whereas the effect of muscular stress imposed by resistance-based exercise has yet to be investigated. To the author's knowledge, there are currently no published data on the effect of concentric muscle loading during isokinetic exercise on salivary metabolites as measured by ^{1}H NMR spectroscopy. Such data may provide indirect markers of systemic responses towards muscular loading and may therefore inform the prescription of resistance-based exercise in sports medicine.

3.2 Chapter Aim

The aim of this Chapter is to investigate the effect of HIE and LIE on saliva metabolites by performing 600 MHz ^{1}H NMR spectroscopy experiments on saliva sampled pre- and post-exercise. Since increasing the number of scans by a multiple of four doubles the signal-to-noise ratio, 256 FIDs (scans) were recorded in this study, above the 64 or 128 scans previously published in the literature (Silwood *et al.*, 2002; Walsh *et al.*, 2006; Grootveld *et al.*, 2009; Bertram *et al.*, 2009; Wongravee *et al.*, 2010; Lemanska *et al.*, 2012), to optimise the signal-to-noise ratio to account for subtle changes in salivary metabolites between pre- and post-exercise samples.

Objectives:

I. Present a ^{1}H NMR spectrum (128 scans) of a water sample exposed to a salivette, to investigate and manage potential interference on NMR signals arising from salivette sample collection.

II. Perform PCA on the ^{1}H NMR spectral datasets to investigate the effect of HIE and LIE on saliva metabolites without a predetermined classifier and detect and manage outlying spectra.

III. Perform PLS-DA on the ^{1}H NMR spectral datasets to optimise separation of scores according to class and elucidate saliva metabolites potentially associated with muscular stress imposed by HIE and LIE.

3.3 Results and Discussion

In this chapter, the 56 saliva samples are each referred to by a unique identification code: the letters ‘x’ or ‘y’ denotes the point of collection for either pre- or post-exercise, respectively; ‘H’ or ‘L’ denotes either the high or low intensity exercise session, respectively, from which the sample was collected; the numbers ‘01-14’, denotes the participant number. However, no data is available for samples yH05 and xL07 due to loss of data; the NMR tube broke containing the sample yH05 and no TSP appeared to present in the sample xL07.

3.3.1 ^{1}H NMR Spectrum of a Water Sample Exposed to a Salivette

To investigate potential interference on ^{1}H NMR spectra arising from salivette sample collection, the spectrum of a pure water sample exposed to a salivette is presented in Figure 3.1. No NMR artefacts (false signals of electronic noise) were detected after 8 scans (Appendix 3.1), which suggests that any signals present after 128 scans were due to salivette interference, since an increased number of scans attenuates artefacts. In agreement with the literature (Walsh *et al.*, 2006; Bertram *et al.*, 2009), the greatest interference from salivette exposure was exhibited in the spectral region of δ 3.00-4.50 (a portion of the aliphatic region). The region of δ 3.00-4.00 of the saliva spectrum contains

multiple signals of glucose molecules and CH protons of amino acids (Silwood *et al.*, 2002). Thus, salivette interference may exaggerate these metabolite signals and question the accuracy of semi-quantification and quantification of glucose and amino acids reported in salivary metabolomic studies which employed the salivette sampling method. In fact, Bertram *et al.* (2009) excluded the region of δ 3.66-3.73 from chemometric statistical analysis in an attempt to remove signals from salivette interference, which fulfilled the aim of their study to discriminate between morning and evening sampling times (diurnal variation).

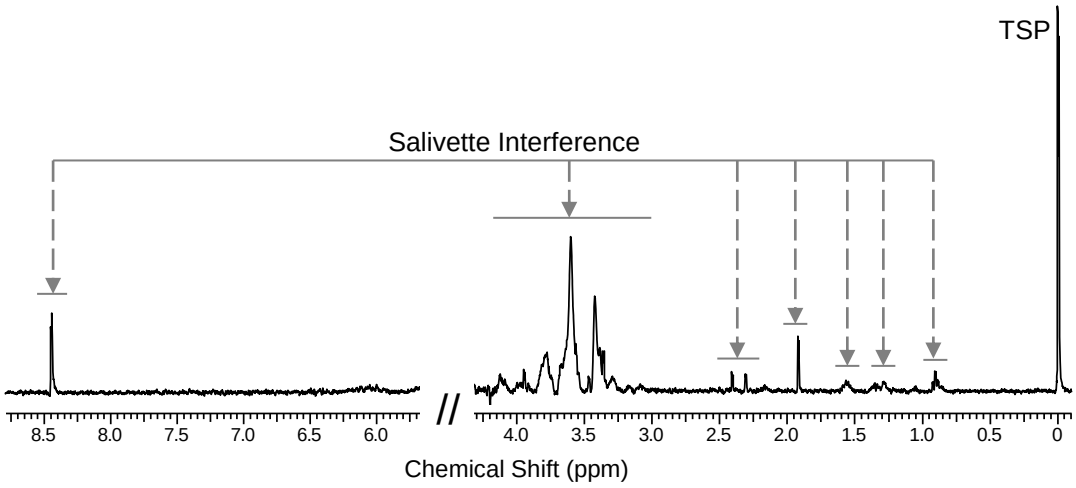


Figure 3.1 400 MHz ^{1}H NMR (1D ZGPR) spectrum (128 scans) of water exposed to a salivette and then treated with TSP as a chemical shift reference. The spectrum shows the aliphatic (δ 0.00-4.25) and aromatic regions (δ 5.75-8.75) to exclude the HDO signal (δ 4.50-5.50). The signals from salivette background noise have been indicated.

However, the findings of the present study elucidated other signals present throughout the aliphatic region and a signal in the aromatic region in the water-salivette spectrum. In particular, distorted signals at δ 1.91-193 and 8.43-8.46 were relatively pronounced and may exaggerate signals of acetate (δ 1.92) and formate (δ 8.46) in the saliva spectrum. Less pronounced broad signals resulting from salivette interference were

present at δ 0.80-0.94, 1.23-1.41, 1.50-1.61, 2.29-2.32 and 2.40-2.42, which may exaggerate a number of metabolite signals. To identify metabolites that may be subject to salivette interference, the chemical shift references of the salivette signals were cross-referenced with signals of saliva metabolites previously detected by ^{1}H NMR spectroscopy in a study that employed expectoration sampling (Silwood *et al.*, 2002) (Table 3.1). Salivettes are made of polypropylene copolymerised with polyethylene for a robust, flexible structure (according to the manufacturer's specification) and such materials contain multiple methyl substituents that are detectable by ^{1}H NMR spectroscopy, which may explain the interference exhibited in a number of signals.

Table 3.1 ^{1}H NMR signals of metabolites that may be subject to salivette interference

^{1}H δ (ppm)	**Multiplicity**	**Assignment**	**Metabolite**
0.83	t	CH_3	*n*-caproate
1.58	m	CH_2	
0.85	t	CH_3	*n*-valerate
1.58	m	CH_2	
0.86	t	CH_3	Saturated fatty acid
1.54	m	$C\underline{H}_2CH_2CO_2R$	
0.89	t	CH_3	*n*-butyrate
1.58	m	CH_2	
0.91	d	CH_3's	Iso-caproate
1.55	m	CH_2	
0.94	t	CH_3	isoleucine
1.92	s	CH_3	Acetate
2.29	t	CH_2	γ-aminobutyrate
2.41	s	CH_2's	Succinate
3.00-4.00	Various	Various	Glucose and amino acids
8.46	s	CH	Formate

Although the use of salivettes is commonplace for targeted analysis in sport and exercise science, and metabolomic data on saliva collected by salivettes has been published previously (Walsh *et al.*, 2006; Bertram *et al.*, 2009; Santone *et al.*, 2014; Ra *et al.*, 2014), the use of such collection products should be approached with caution to avoid misinterpretation from exaggerated signal intensities, particularly in the region of δ 3.00-4.50. Thus, alternative methods of saliva collection for ^{1}H NMR spectroscopy should be considered in future studies, such as expectoration (Silwood *et al.*, 2002) or passive drool (Takeda *et al.*, 2009).

3.3.2 PCA of ^{1}H NMR Spectral Data of Saliva: Outlier Detection and Management

PCA was performed on the 600 MHz ^{1}H NMR spectra to investigate the effect of isokinetic exercise on saliva metabolites without the influence of a predetermined classifier (unsupervised) and to detect potential outlying spectra, since outliers can mathematically skew data models and usually fail to provide information coherent with representative scores. The saliva dataset (excluding the dark regions TSP and HDO) was normalised to the sum of intensities to account for differences in saliva volumes collected and concentrations between samples. Resonance assignment was carried out based on chemical shift values and signal multiplicity according to the literature (Silwood *et al.*, 2002) and the Human Metabolome Database (www.hmdb.ca).

Figure 3.2A presents the PCA scores plot of the HIE dataset, whereby PC1 and PC2 explain 48.2% and 18.6% of variance, respectively. The score xH12 fell outside of Hotelling's 95% confidence limits (ellipses) and was located in the upper-left quadrant. The loadings plot presented in Figure 3.2B elucidated the spectral integrals in the upper-left quadrant as the variable weightings on the outlying score. Consistent with the loadings, visual inspection of spectrum xH12 showed that multiple signals within the region of δ 3.00-4.00 were more pronounced than xL12, a benchmark sample taken at rest from the same participant on a different occasion which fell within Hotelling's 95% confidence ellipses of the LIE dataset (Figure 3.3). This particular region has been associated with salivette interference as

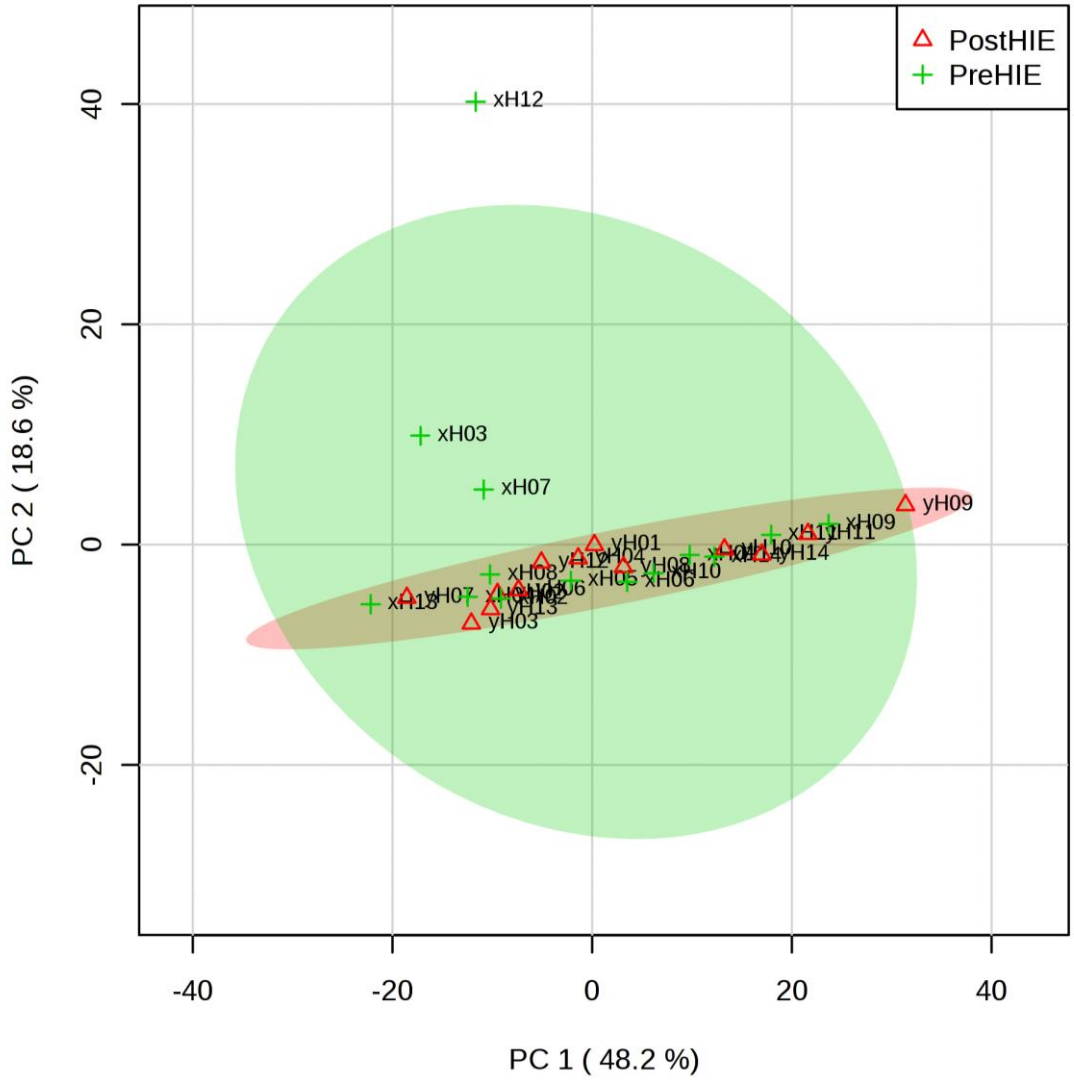


Figure 3.2A HIE Supervised Model: PCA scores plot of 600 MHz ^{1}H NMR spectra of saliva, normalised to sum of intensities, taken from 14 healthy men (+) pre- and (Δ) post-HIE. PC1 and PC2 explain 48.6% and 18.6% of variance, respectively. The score xH12 fell outside of Hotelling's 95% confidence ellipses. Throughout this chapter, the green and red ellipses in all data models denote the 95% confidence regions for the pre- and post-exercise scores, respectively.

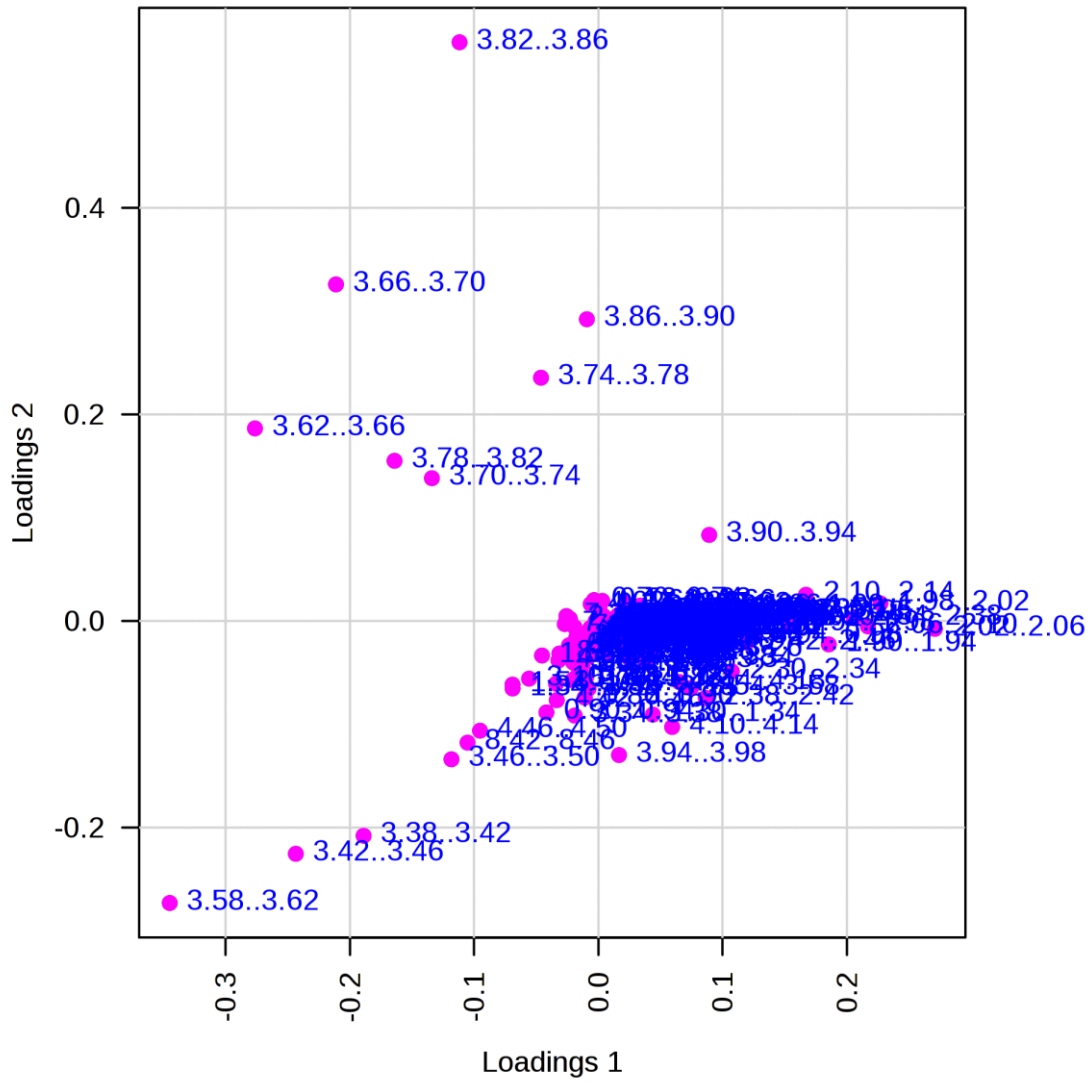


Figure 3.2B HIE Unsupervised Model: PCA loadings plot showing the weighting of variables (spectral integrals) on the HIE scores. All integrals that separated from the cluster at zero contain resonances which may have been subjected to salivette interference according to the results of the salivette-water spectrum (Figure 3.1).

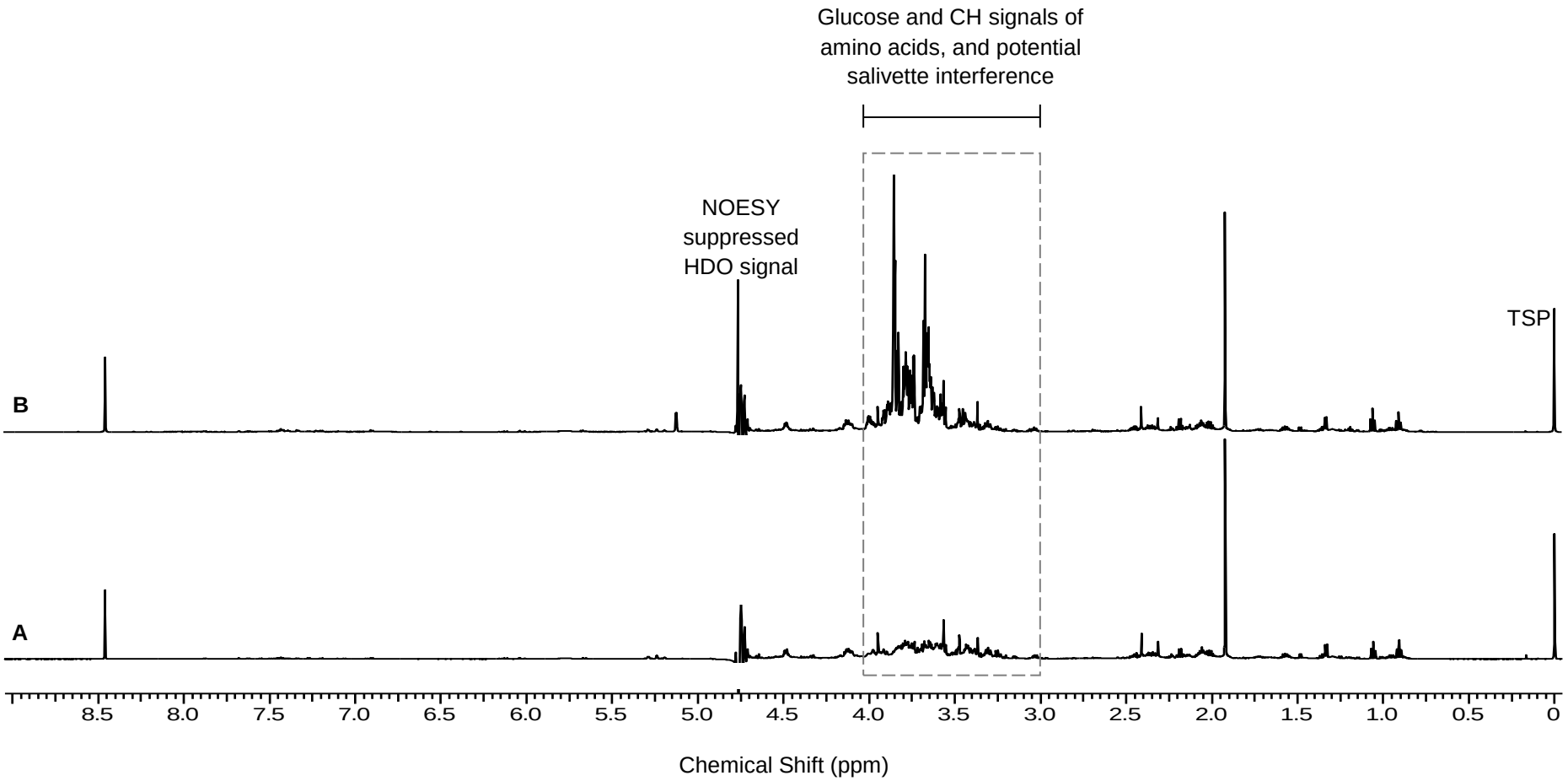


Figure 3.3 600 MHz ^{1}H NMR spectra of saliva (256 scans) taken pre-exercise (at rest) from participant 12 on two separate occasions; **(A)** benchmark spectrum which fell within Hotelling's 95% confidence limits of the data model (for comparison) and **(B)** the outlying spectrum. The signals within the region of δ 3-4 are noticeably more pronounced in spectrum (B), possibly due to salivette exposure.

demonstrated in Figure 3.1 and suggested in the literature (Walsh *et al.*, 2006; Bertram *et al.*, 2009).

The PCA scores plot of the LIE dataset is presented in Figure 3.4A whereby PC1 and PC2 explain 53.7% and 15.1% of variance, respectively. The scores xL09 and yL09 (same individual) were located on the border-line of Hotelling's 95% confidence ellipses. Consistent with the loadings (Figure 3.4B), an unusual pronounced singlet was present in the spectra xL09 and yL09 at δ 3.56 (Figure 3.5), which may be attributable to interference from a dental hygiene product as there was no reference according to the literature and HMDB. Acetate (δ 1.92) and formate (δ 8.46), and potential salivette interference also dominated the loadings.

To manage the outlying scores and suppress potential interference from salivette exposure, the spectral region of δ 3.00-4.50 was deleted from the entire dataset and as a result, no outliers were detected in the refined data models since all scores fell within Hotelling's 95% confidence ellipses (Appendix 3.2).

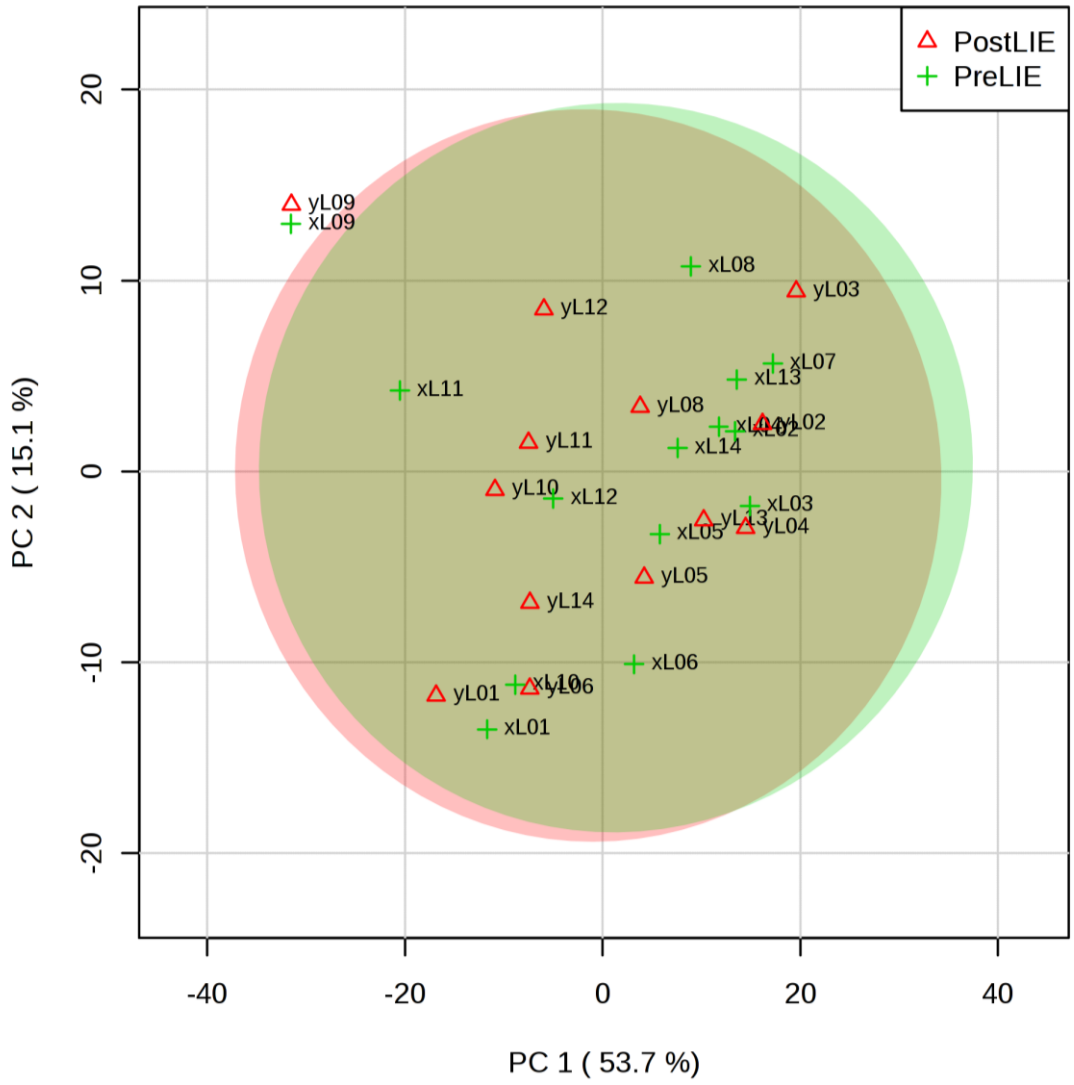


Figure 3.4A LIE Unsupervised Model: PCA scores plot of 600 MHz ^{1}H NMR spectra of saliva, normalised to sum of intensities, taken from 14 healthy men (+) pre- and (Δ) post-LIE. PC1 and PC2 explain 53.7% and 15.1% of variance, respectively. The pre- and post-LIE scores for participant 09 fell slightly outside of Hotelling's 95% confidence ellipses.

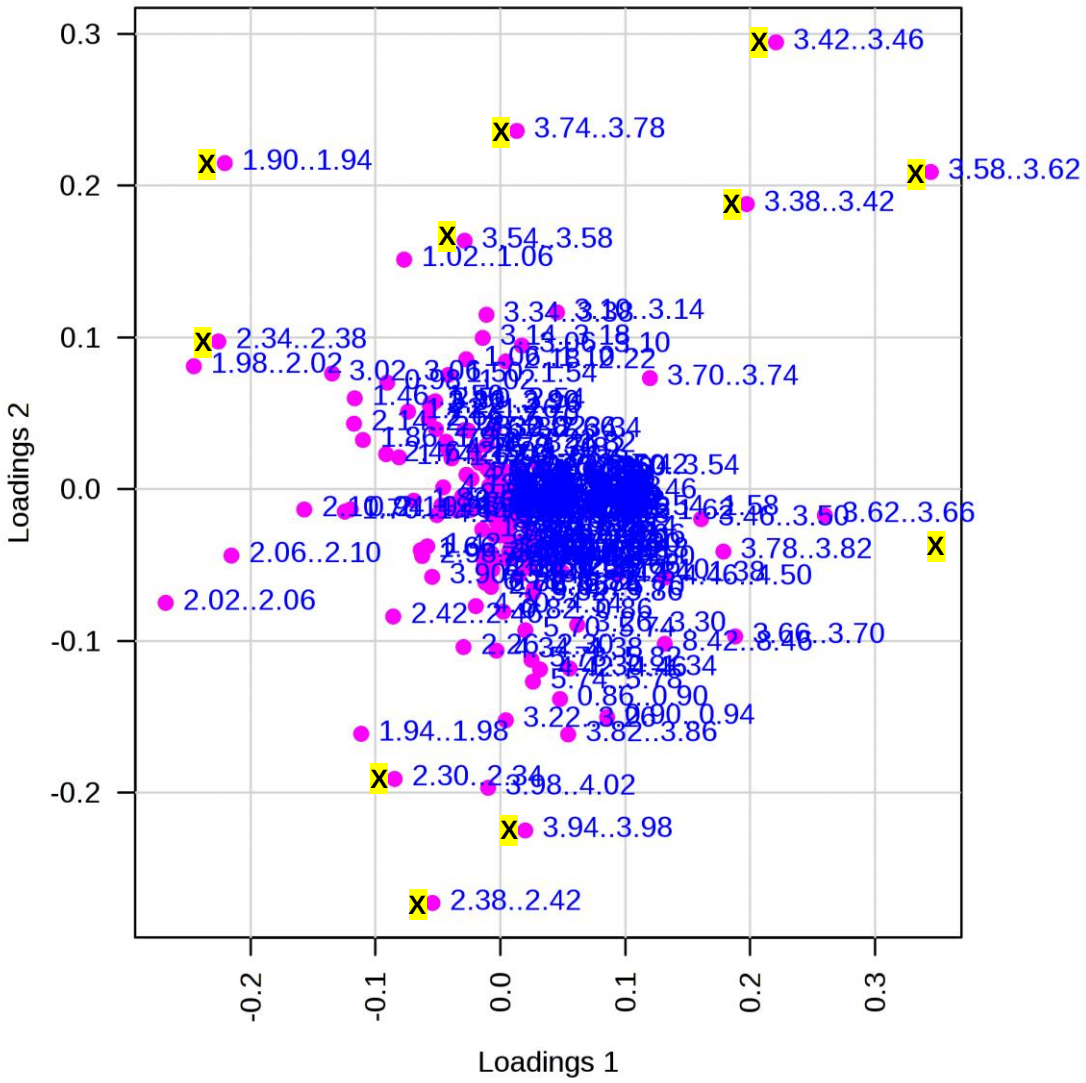


Figure 3.4B LIE Unsupervised Model: PCA loadings plot showing the weighting of variables (spectral integrals) on the LIE scores. 'X' marks the integrals that separated from the cluster at zero which contain signals which may have been subjected to salivette interference according to the results of the salivette-water spectrum (Figure 3.1).

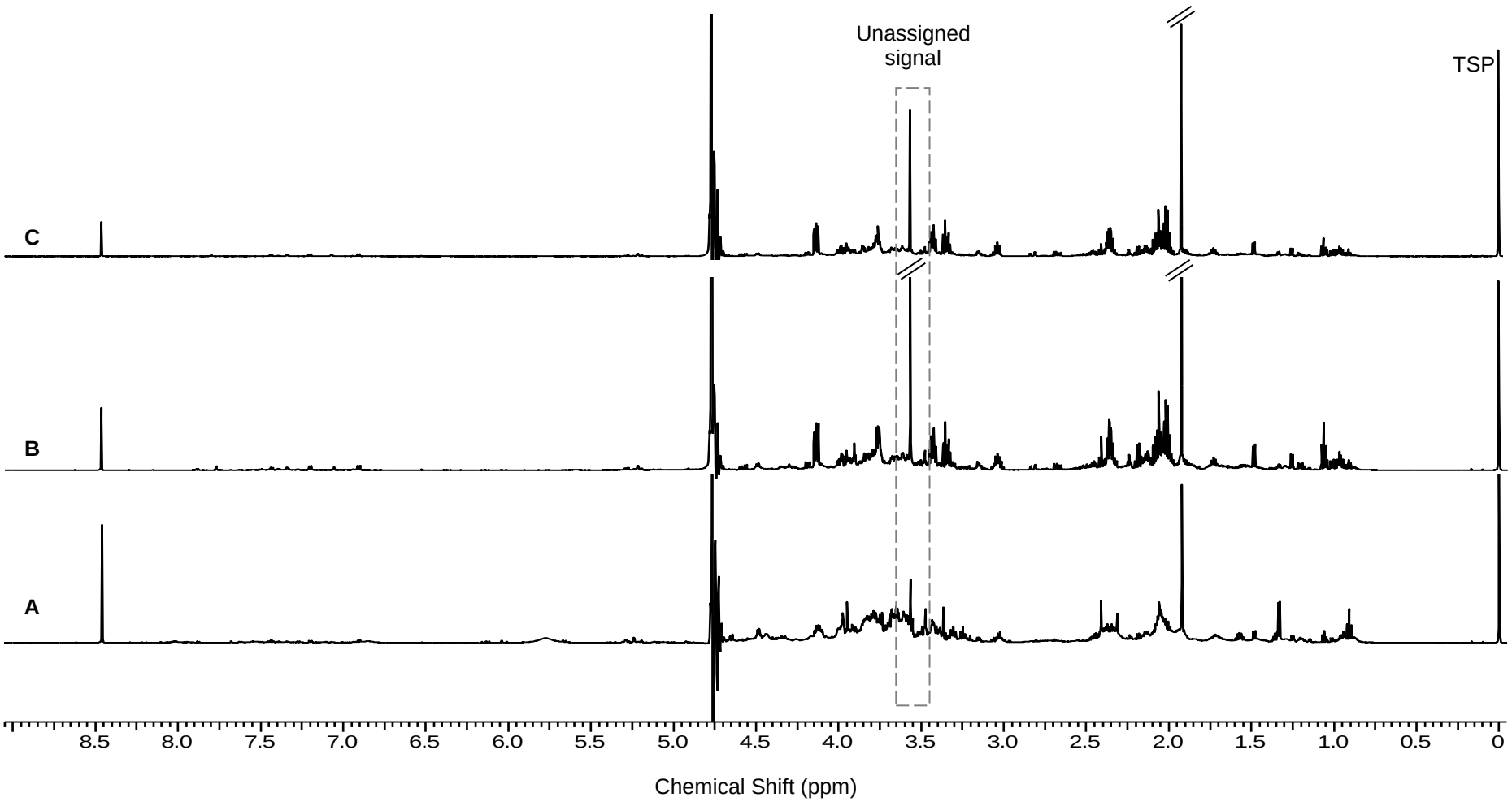


Figure 3.5 600 MHz ^{1}H NMR spectra of saliva (256 scans) taken from participant 09: **(A)** Pre-HIE (a benchmark sample that fell within Hotelling's 95% confidence ellipses of the HIE data model); **(B)** pre- and **(C)** post-LIE (outlying spectra).

3.3.3 The Effect of HIE and LIE on Saliva Metabolites after Model Refinement

PLS-DA was performed on the 600 MHz ^{1}H NMR spectra of saliva to discriminate between classes with exercise as the classifier (supervised) and to elucidate spectral integrals associated with the classifier to inform resonance assignment, after the deletion of the spectral region of δ 3.00-4.50 in an attempt to reduce salivette interference.

Figure 3.6A presents the PLS-DA sores plot of the HIE dataset, whereby component 1 and 2 explain 41.5% and 21% of variance, respectively. A partial class distinction was achieved since the majority of scores separated according to the classifier, with a minor overlap of scores (yH06, yH10, yH12 and yH14). Inter-individual variation was high since the separation between scores varied depending on the individual; the greater the separation between individual scores (pre- and post-HIE), the greater the metabolic heterogeneities according to the classifier. The PLS-DA loadings plot presented in Figure 3.6B illustrates that the strongest variable weightings (according to the classifier) on the HIE scores were integrals containing signals assigned to *n*-butyrate (δ 0.89, 1.58 and 2.15), ethanol (δ 1.21), lactate (δ 1.33), acetate (δ 1.92), *N*-acetyl sugars (δ 2.05) and formate (δ 8.46).

By comparison, the PLS-DA scores plot of the LIE dataset (Figure 3.7A), which explains 36.4% and 28.1% of variance across component 1 and 2, respectively, exhibited a slightly weaker class separation since more scores overlapped. According to the loadings plot presented in Figure 3.7B, a trend was exhibited in both the HIE and LIE data models in terms of the variable weightings, with the exception of the acetate integral which was located in the upper-right quadrant and lower-left quadrant of the LIE and HIE model, respectively. Illustrative spectra of saliva obtained from one individual are presented in Figure 3.8 to illustrate resonance assignment.

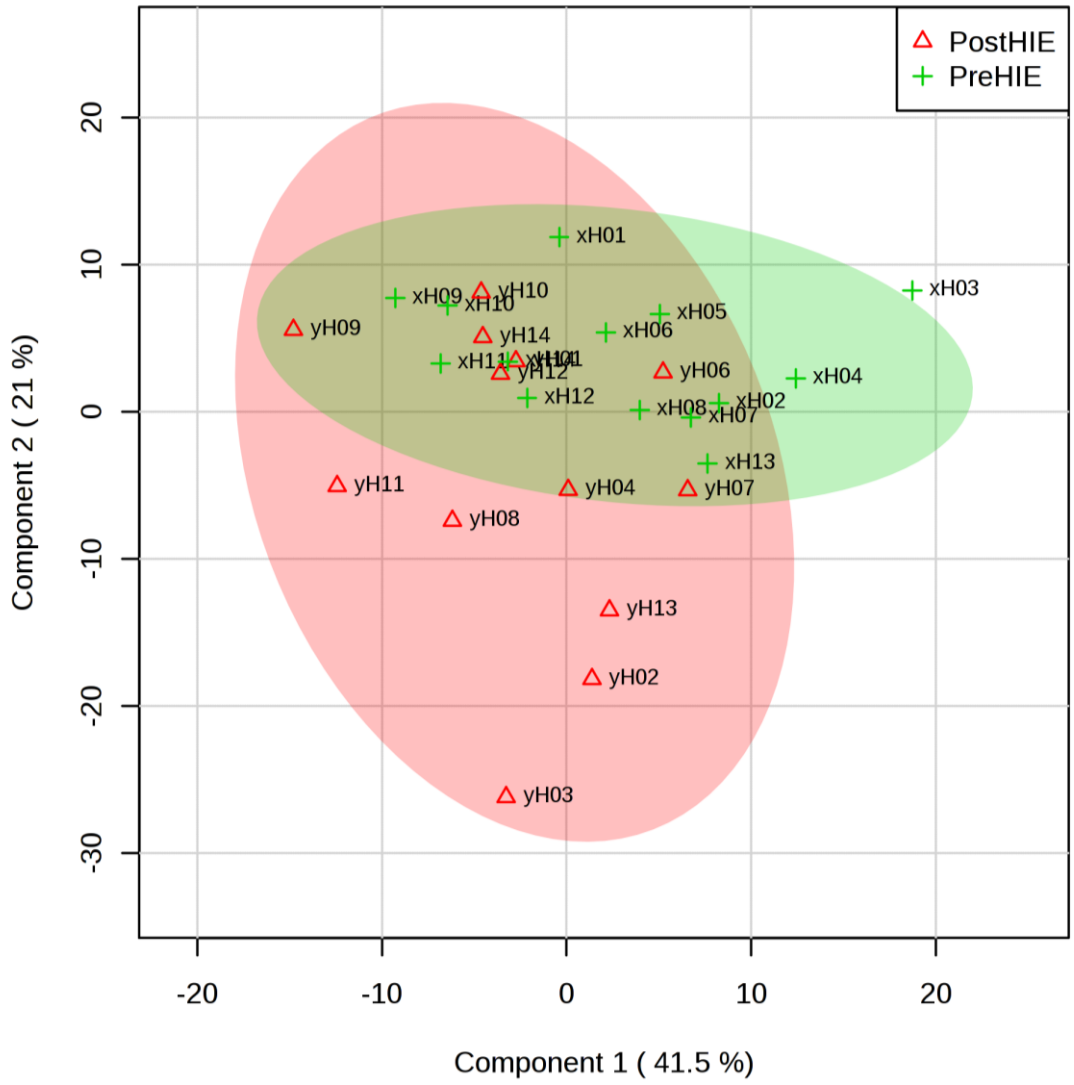


Figure 3.6A HIE Supervised Model: PLS-DA scores plot of 600 MHz ^{1}H NMR spectra of saliva (excluding the region of δ 3.00-4.50), normalised to sum of intensities, taken from 14 healthy men (+) pre- and (Δ) post-HIE. PC1 and PC2 explain 41.5% and 21% of variance, respectively. A partial class distinction was established due to score overlap between class clusters.

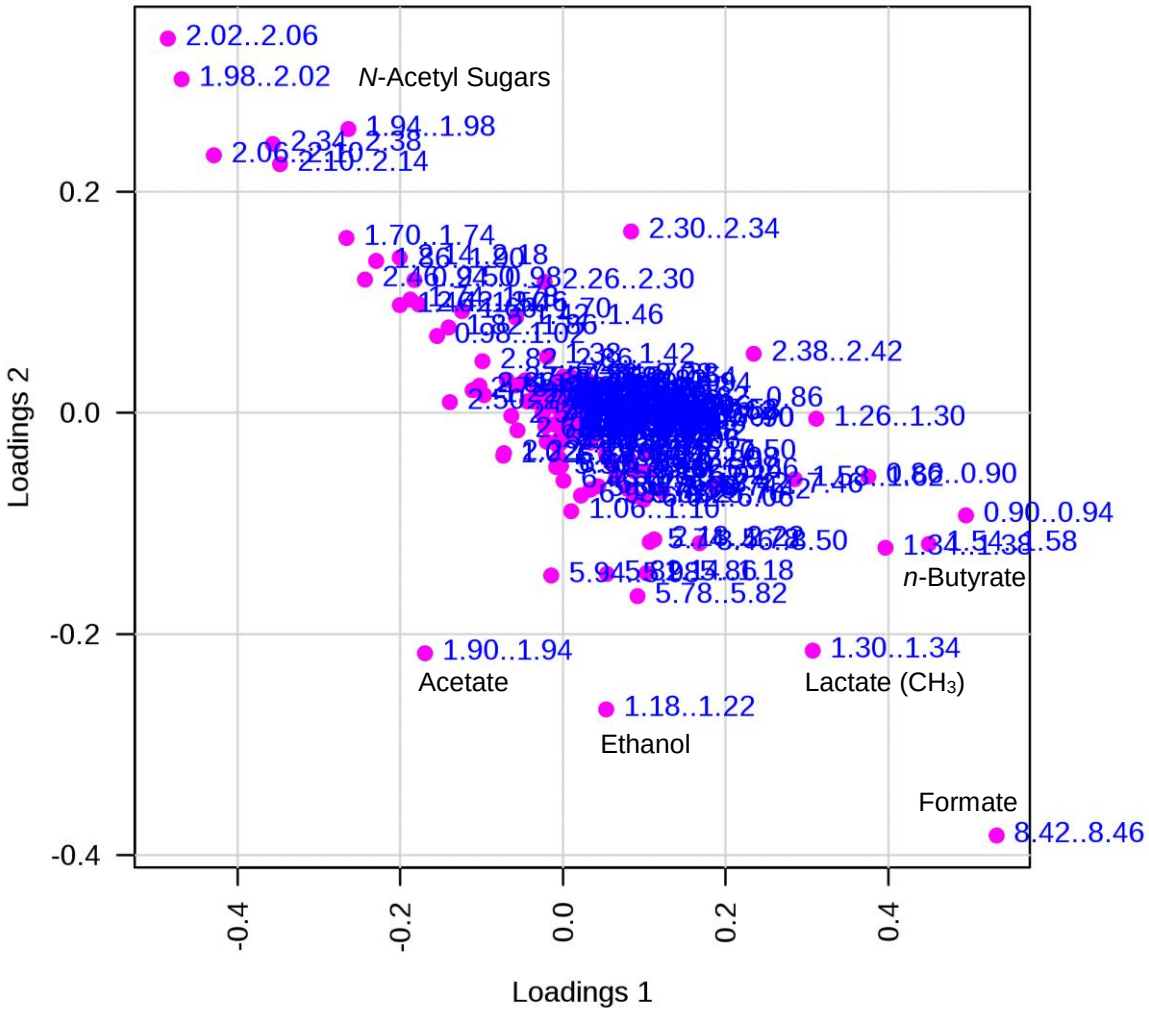


Figure 3.6B HIE Supervised Model: PLS-DA loadings plot showing the weighting of variables (spectral integrals) on the HIE scores excluding the region of δ 3.00-4.50. Integrals that separated from the cluster at zero were assigned to *n*-butyrate (δ 0.89, 1.58 and 2.15), ethanol (δ 1.21), lactate (δ 1.33), acetate (δ 1.92), *N*-acetyl sugars (δ 2.05) and formate (δ 8.46).

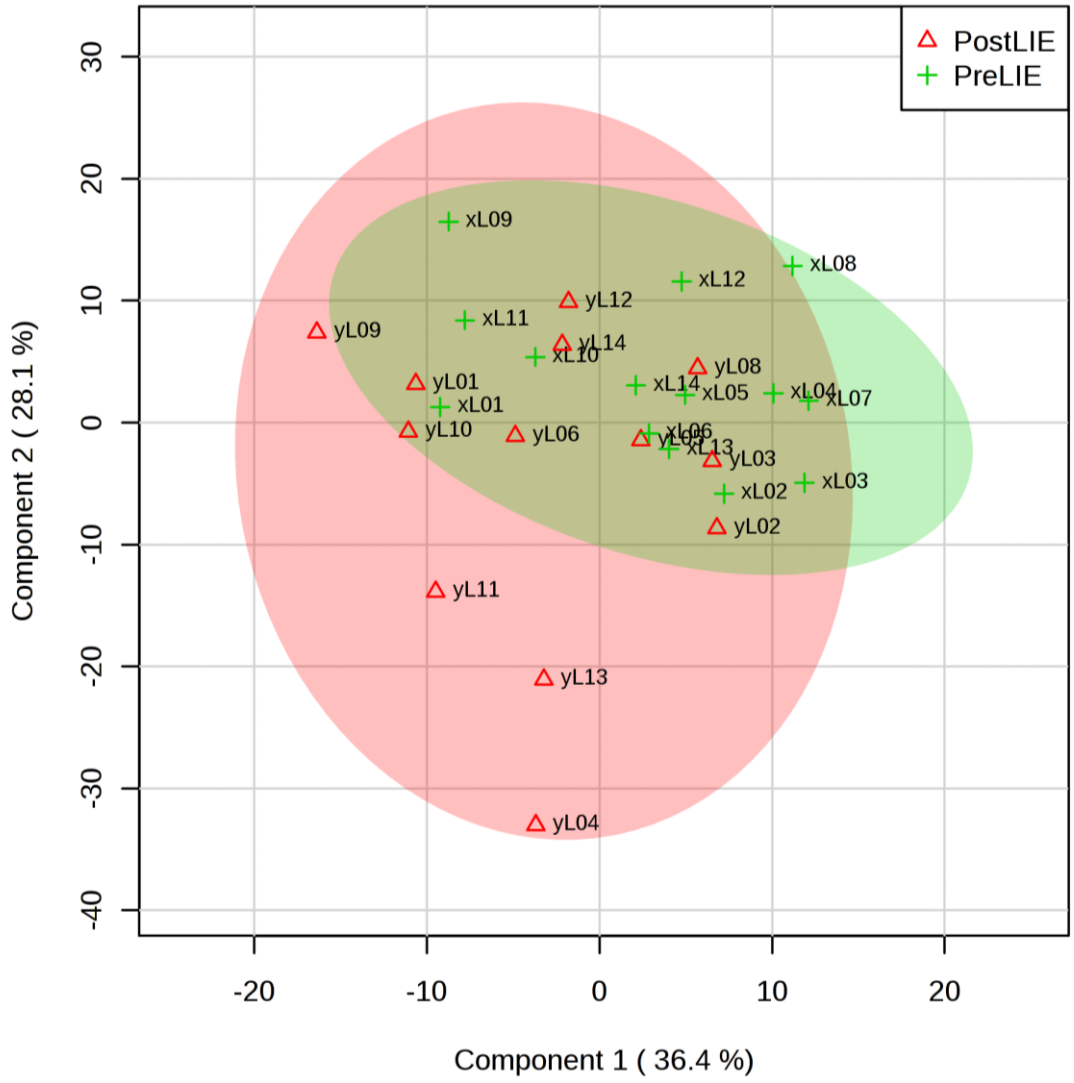


Figure 3.7A LIE Supervised Model: PLS-DA scores plot of 600 MHz ^{1}H NMR spectra of saliva (excluding the region of δ 3.00-4.50), normalised to sum of intensities, taken from 14 healthy men (+) pre- and (Δ) post-LIE. PC1 and PC2 explain 36.4% and 28.1% of variance, respectively. A partial class distinction was established due to score overlap between class clusters.

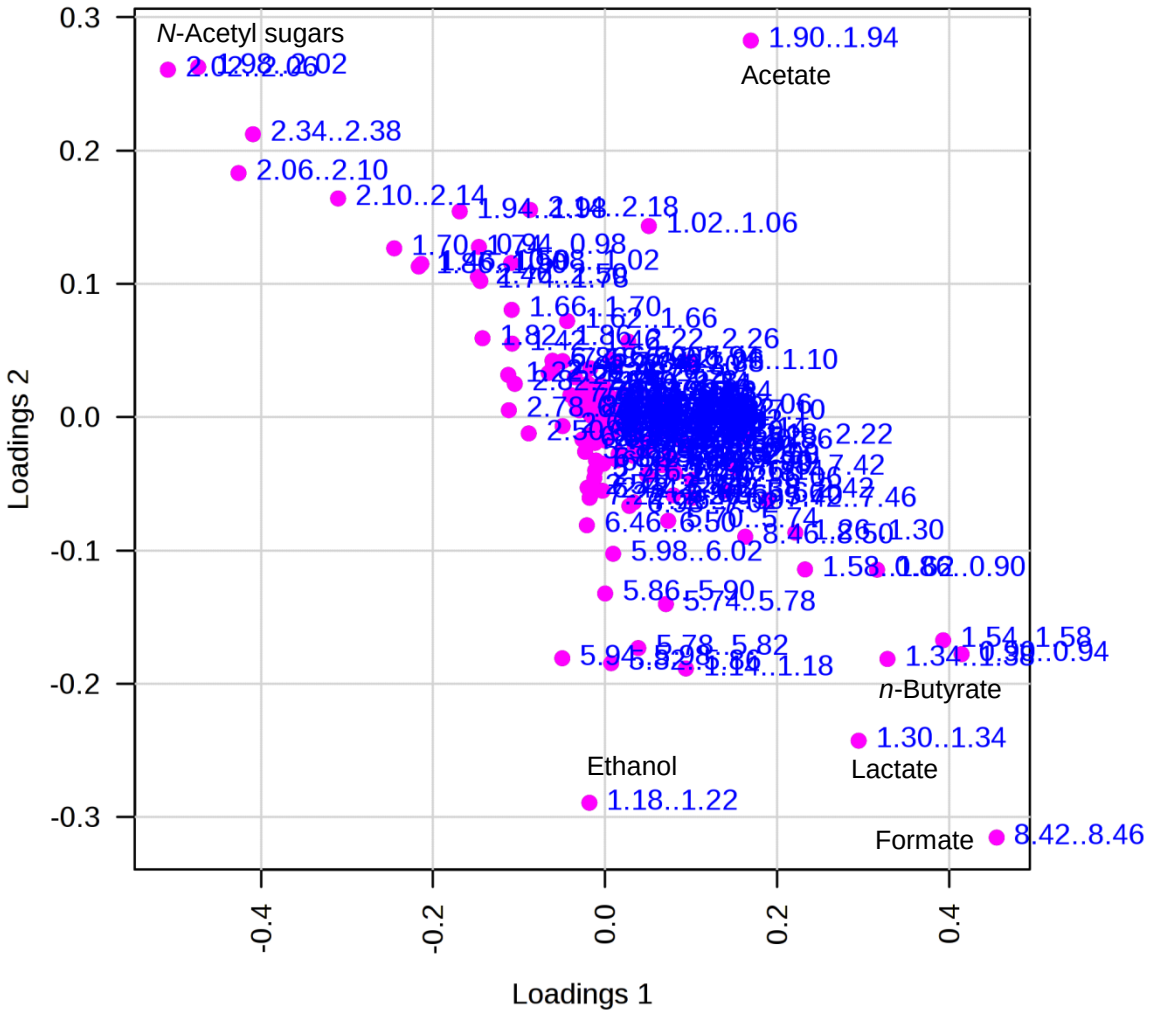


Figure 3.7B LIE Supervised Model: PLS-DA loadings plot showing the weighting of variables (spectral integrals) on the LIE scores excluding the region of δ 3.00-4.50. Integrals that separated from the cluster at zero were assigned to *n*-butyrate (δ 0.89, 1.58 and 2.15), ethanol (δ 1.21), lactate (δ 1.33), acetate (δ 1.92), *N*-acetyl sugars (δ 2.05) and formate (δ 8.46).

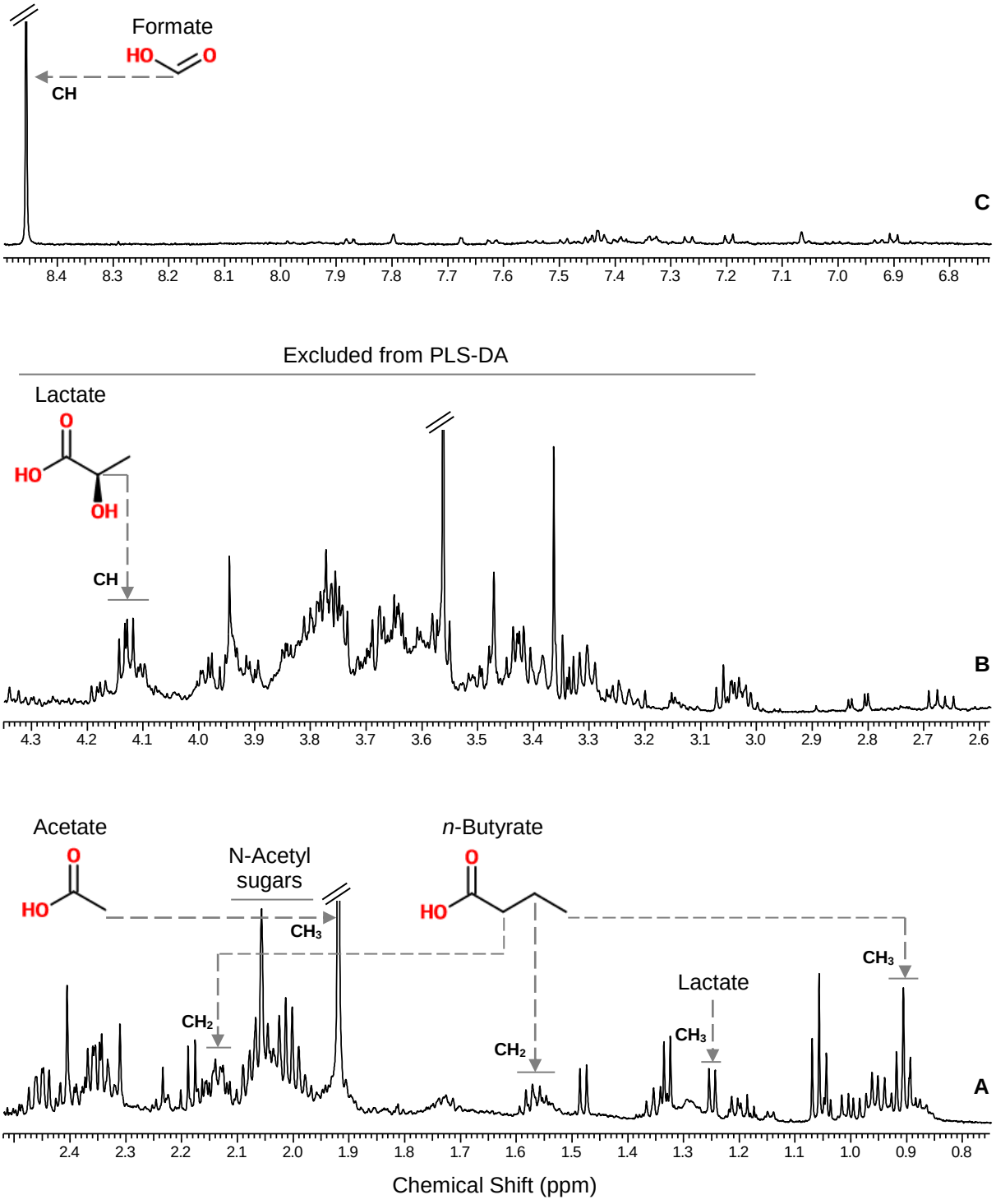


Figure 3.8 600 MHz ^{1}H NMR spectrum of saliva taken at rest from a participant, presented in the three expanded regions of **(A)** δ 0.80-2.50, **(B)** 2.60-4.30 and **(C)** 6.80-8.50. Signals elucidated by PLS-DA were assigned to short-chain organic acid anions and *N*-acetyl sugars as labelled. The CH signal of lactate is also illustrated to provide full assignment to the molecular structure, despite the subsequent removal of δ 3.0-4.5 from integral dataset in an attempt to resolve salivette interference.

Since the inter- and intra-variability was high amongst the participants, no uniform change in magnitude or direction of the values of salivary lactate, acetate and formate was exhibited in the HIE and LIE datasets (Table 3.2). Marked increases have been reported in salivary lactate concentration from approximately 0.25-4.25 mM following incremental swimming (Bocanegra *et al.*, 2012) and 0.25-3 mM following incremental cycling (Segura *et al.*, 1996), as measured by electro-enzymatic assay (Biochemical Analyzer YSI, Ohio, USA). The results of the present study contradict the findings of these previous studies, since a 2.25 and 1.35 fold-decrease in salivary lactate was exhibited in 7-8 individuals following HIE and LIE, respectively. A possible reason for this disagreement was that the studies published in the literature recruited swimmers and athletes with the same training backgrounds, whereas the present study recruited a less homogenous population sample of healthy individuals, hence the inter-variability exhibited in metabolic responses to isokinetic exercise. Santone *et al.* (2014) reported that salivary lactate, acetate and formate were associated with metabolic perturbations induced by the Yo-Yo Test (running intervals), as measured by ^{1}H NMR spectroscopy, yet no semi-quantitative values in fold change were documented for comparison to the present study.

Table 3.2 Semi-quantitative fold changes from pre- to post-exercise of the metabolite integrals, elucidated by PLS-DA, normalised to the TSP signal (mean ± SD; *n*=number of individuals).

Metabolite	**HIE Dataset (Fold Change)**	**LIE Dataset (Fold Change)**
Lactate (δ 1.33)	↑ 2.25 ± 1.51 (*n*=5)	↑ 2.13 ± 0.96 (*n*=6)
	↓ 2.52 ± 0.92 (*n*=8)	↓ 1.35 ± 0.30 (*n*=7)
Acetate (δ 1.92)	↑ 1.96 ± 0.87 (*n*=6)	↑ 1.54 ± 0.49 (*n*=5)
	↓ 2.55 ± 0.98 (*n*=7)	↓ 1.43 ± 0.38 (*n*=8)
Formate (δ 8.46)	↑ 1.86 ± 1.00 (*n*=6)	↑ 1.39 ± 0.13 (*n*=6)
	↓ 2.51 ± 0.45 (*n*=7)	↓ 1.38 ± 0.36 (*n*=7)

No numerical values were provided for *n*-butyrate, *N*-Acetyl sugars and ethanol due to signal overlap.

Acetate, *n*-butyrate, lactate and formate are anions of short-chain organic acids (acetic acid, butyric acid, lactic acid and formic acid, respectively) which, in saliva, are mainly metabolites produced from the fermentation of carbohydrates by bacteria such as *Streptococcus* (Silwood *et al.*, 2002). Since the bacterial content of saliva differs between individuals (Lemanska *et al.*, 2012), the variability in these short-chain organic acid signals may have been influential on the inter-individual variation exhibited in the PCA and PLS-DA model of the HIE and LIE datasets. The resonances of *N*-Acetyl sugars detected in saliva are also likely to be derivatives of bacterial metabolism via the enzymes hyaluronidase and neuraminidase (Silwood *et al.*, 2002). Although ethanol is an exogenous metabolite that may be present in saliva after the consumption of alcoholic drinks, concentrations of salivary ethanol may increase following carbohydrate metabolism in bacteria such as *Streptococcus mutans* (Silwood *et al.*, 2002).

Acetate together with glucose are derivatives and precursors of acetyl CoA, involving the enzymes acetyl CoA hydroxylase and acetyl CoA synthase, respectively, in a two-way conversion pathway (Pouteau *et al.*, 1996). Acetate present in blood plasma has been reported to contribute towards 6.5% of basal energy expenditure via uptake in skeletal muscles (Pouteau *et al.*, 1996) and a 6-fold increase in uptake from 10 to 60 $\mu mol.min^{-1}$ was reported following knee extension exercise (van Hall *et al.*, 2002), similar to the intervention employed in the present study. However, acetate present in the blood and skeletal muscle originates from the metabolism of bacteria such as *Bacteroides*, *Eubacterium* and *Peptostreptococcus*, within the gastrointestinal tract (Bergman, 1990), whereas salivary acetate originates from salivary bacteria and thus, it is unlikely that salivary acetate and other short-chain organic acids were directly related to muscle energetics in the present isokinetic exercise study, considering the biological origin. Nevertheless, the salivary content of acetate, lactate and formate are influenced by salivary gland stimulation (Takeda *et al.*, 2009) and hence, the sympathetic-adrenal response to muscular exercise (Seals and Victor, 1991) may have been indirectly influential on the secretion of these metabolites.

3.4 Chapter Conclusion

The potential of saliva, via salivette collection, as a non-invasive matrix to study the metabolic effects of exercise by ^{1}H NMR spectroscopy has been demonstrated previously (Santone *et al.*, 2014). However, this chapter demonstrates that saliva analysis in an isokinetic exercise setting has limitations relating to the salivette collection method, inter-individual variation exceeding the effects of exercise in the data models and the origin of salivary metabolites is unclear.

The salivette collection method is commonplace in exercise science and has the advantage of promoting convenient, hygienic sampling with a low risk of spillage. Nevertheless, the results from a ^{1}H NMR experiment on a water sample exposed to a salivette revealed interference in a number of signals throughout the spectrum, beyond the affected spectral region of δ 3.00-4.00 documented previously (Walsh *et al.*, 2006; Bertram *et al.*, 2009). In an attempt to overcome the bulk of salivette interference post-NMR analysis, the spectral region of δ 3.00-4.50 was deleted from the entire dataset, which consequently resulted in data loss on signals of glucose and CH resonances of amino acids. Thus, the use of expectoration or passive drool saliva collection methods should be considered in prospective studies to avoid salivette interference.

PLS-DA of the saliva metabolomic datasets elucidated short-chain organic acids (lactate, acetate, *n*-butyrate and formate) and *N*-acetyl sugars as the strongest variable weightings associated with the classifier; however, the biological origin of these metabolites is equivocal. Short-chain organic acids and *N*-acetyl sugars are derivatives of bacterial metabolism (Silwood *et al.*, 2002) and the use of dental hygiene products affect the microbial content of saliva (Lemanska *et al.*, 2012), which may have influenced the results of this study (prior to the addition of sodium azide). Alternatively, acetate, lactate and formate concentrations are subject to change via salivary gland stimulation (Takeda *et al.*, 2009) and muscular exercise has been previously associated with sympathetic-adrenal responses via the autonomic nervous system (Seals and Victor, 1991). Moreover, the water spectrum

exposed to a salivette gave rise to signals in the regions of δ 0.80-0.94, 1.50-1.61, 1.91-193 and 8.43-8.46 which may have caused interference with *n*-butyrate, acetate and formate signals. A further recommendation for future salivary metabolomic studies on exercise is to provide the participants with standardised dental hygiene products (e.g. toothpaste and/or mouth wash) and instructions on usage during the evening and morning prior to saliva collection in an attempt to avoid potential inter-individual variation in salivary microbial content.

CHAPTER 4: ^{1}H NMR SPECTROSCOPY RESULTS OF URINE SAMPLES AND CHEMOMETRIC MODELLING OF HIE AND LIE

4.1 Background

Muscular stress imposed by resistance-based exercise induces cellular and biomolecular changes, such as protein catabolism, within the working muscle and the eccentric loading phase induces muscle micro-trauma (Nikolaidis *et al.*, 2008; Brancaccio *et al.*, 2010; Brentano and Martins Kruel, 2011). The metabolites that accumulate in muscle and blood plasma after protein degradation include urea, amino acids (Viru and Viru, 2001), and 1-/3-methylhistidine comprised of histidine bound to a CH_3 group (Sampson *et al.*, 2014), all of which are excreted in the urine and measurable by ^{1}H NMR spectroscopy (Pechlivanis *et al.*, 2010). However, the interpretation of such biomarkers should be approached with caution since histidine and 1-/3-methylhistidine are susceptible to ^{1}H signal shifts induced by urinary pH differences (Pechlivanis *et al.*, 2010), which can vary between pH 4.8-7.5 (Stedman, 2000), and urea protons give rise to a broad signal at δ 5.80 due to proton exchange with HDO.

Plasma biomarkers of muscular stress include the proteins CK and myoglobin as measured by enzyme-linked immunoabsorbant assay (Speranza *et al.*, 2007). At the occurrence of rhabdomyolysis, severe skeletal muscle micro-trauma caused by eccentric loading during muscular exercise or injury, myoglobin is released from the site of injury into the blood stream (Warren *et al.*, 2002; Brancaccio *et al.*, 2010). Plasma myoglobin is filtered by the kidneys and may be present in the urine when plasma myoglobin concentration exceeds 1.5 mg/dL, a stage at which myoglobinuria develops which may cause acute renal injury (Subramanian *et al.*, 2013). A case-study of a patient with reversible renal tubular injury from rhabdomyolysis, caused by a side effect of medical treatment for dyslipidemias with statins, reported a marked increase in urinary excretion of dimethylamine and

trimethylamine-*N*-oxide, and a decrease in glycine, hippurate and citrate as measured by 400 MHz ^{1}H NMR spectroscopy (Bairaktari *et al.*, 2002). This suggests that detecting such change in the urinary excretion of these metabolites after muscular exercise may reflect imposed renal stress, possibly from renal filtration of plasma myoglobin released from muscle tissue during eccentric loading (micro-trauma).

It is evident from recent NMR-based metabolomic studies on urine that sprint running may indirectly impose renal stress based on the reported change in urinary excretion of citrate, glycine, hippurate and trimethylamine *N*-oxide (Pechlivanis *et al.*, 2010), whereas cycling may not (Enea *et al.*, 2010; Mukherjee *et al.*, 2014). It is possible that the eccentric muscle loading induced by sprint running (Baron *et al.*, 2009) may impose muscle micro-trauma which increases the renal filtration of myoglobin, whereas the continuous motion pattern of cycling (alternated lower-limb pushing actions) imposes concentric loading, hence no muscle micro-trauma. By contrast, Speranza *et al.* (2007) reported a marked increase in plasma myoglobin concentration (from 1.44 to 2.24 mg/dL) following concentric muscle contractions of the knee extensor and flexor muscles via isokinetic exercise, which suggests that concentric muscle loading may indirectly impose renal stress however, no change was reported in plasma CK. To the author's knowledge, there are currently no published data on the effect of concentric muscle loading during isokinetic exercise on urinary metabolites as measured by ^{1}H NMR spectroscopy. Such data may be indicative of indirect renal stress imposed by the filtration of myoglobin released during concentric muscle loading and thus, may inform exercise prescription for clinical or injured populations.

In sports medicine, the urine matrix may provide diagnostic and prognostic information on injured and recovering athletes to determine whether or not they are fit to play, and predict risk of injury based on their current state of health. At present, urinalysis is commonly used for drug doping in sport to encourage fair play amongst contenders. Physiological determinants of health and fitness, and athletic status are well established in sports practice, such as measurements of heart rate, blood pressure, BMI, aerobic and

anaerobic capacity, muscular strength and targeted blood biomarkers (cortisol, testosterone, myoglobin, CK and other stress proteins, lactate, glucose and cholesterol). However, urinalysis by ^{1}H NMR spectroscopy to determine biomarkers of exercise tolerance has yet to progress from sports research to sports practice, possibly because of the impracticality of urine storage and transportation in a sports setting, and access to such bioanalytical technology.

A limitation of urinalysis in exercise science is that biomarkers have different, and in some cases multiple, biological origins (Sampson *et al.*, 2014) and thus, urinary constituents (pooled) may only serve as indirect biomarkers of muscle metabolism and direct biomarkers of renal function. Urinary creatinine excretion is a prime example of a biomarker linked to both muscle mass (Slupsky *et al.*, 2007) and glomerular filtration rate (rate at which the blood waste products are filtered by the glomerulus which ranges between 90-120 mL.min^{-1}), representative of renal function (Waikar *et al.*, 2010). The renal processes glomerular filtration, tubular reabsorption and secretion serve the following functions: Maintain hydration and osmolality; regulate balance of salts/ions (sodium, potassium, calcium, magnesium, chloride and bicarbonate) and pH; filter metabolic end-products from the blood and dissolve the waste products in the urine for disposal (Rhoades and Bell, 2009). Thus, the concentration of urinary solutes is primarily dependent on the aforementioned renal processes and hydration status of the individual.

The diagnostic value of urine as a non-invasive, biofluid matrix in NMR spectroscopy-based studies has been well documented in renal (Nicholson *et al.*, 1985; Gartland *et al.*, 1990; Bairaktari *et al.*, 2002; Posada-Ayala *et al.*, 2014), hepatic (Ladep *et al.*, 2014) and metabolic pathology (Nicholson *et al.*, 1984; Holmes *et al.*, 1997; Stamler *et al.*, 2013). However, urinary metabolomics (NMR- or GC/LC-MS-based) in exercise physiology is in its infancy and recent studies have focused on profiling metabolic perturbations induced by cycling (Enea *et al.*, 2010; Mukherjee *et al.*, 2014) and sprint running (Pechlivanis *et al.*,

2010), or a combination of the two (Daskalaki *et al.*, 2015), whereas the effect of muscular stress imposed by resistance-based exercise has yet to be investigated.

To investigate the effect of HIE and LIE on urinary metabolites, there are a number of challenges to overcome: Standardising dietary intake and water consumption prior to participation; accounting for differences in urinary concentration (osmolality) and pH between samples; the influence of the intense creatinine signals (δ 3.05 and δ 4.06) and the broad urea signal (δ 5.80) of urine spectra on data models; reduction and interpretation of complex multivariate datasets to elucidate metabolites associated with the exercise interventions employed. This chapter will attempt to address a number of these challenges.

4.2 Chapter Aim

The aim of this chapter is to investigate the effect of HIE and LIE on urinary metabolites by performing 600 MHz ^{1}H NMR spectroscopy experiments on urine sampled pre- and post-exercise.

Objectives:

I. Report urinary pH and osmolality values of samples as descriptive information concerning pH and concentration differences between urine samples.

II. Perform PCA on the ^{1}H NMR spectral datasets to investigate the effect of HIE and LIE on urinary metabolites without a predetermined classifier and detect and manage outlying spectra.

III. Determine whether data normalisation to sum of intensities or normalisation to the creatinine signal at δ 3.05 provides the most robust chemometric models of the datasets in terms of variance explained across PC1 and PC2.

IV. Perform PLS-DA on the ^{1}H NMR spectral datasets to optimise separation of scores according to class and elucidate metabolites potentially associated with muscular stress imposed by HIE and LIE.

4.3 Results and Discussion

In this chapter, the 56 urine samples are each referred to by a unique identification code: the letters ‘x’ or ‘y’ denotes the point of collection for either pre- or post-exercise, respectively; ‘H’ or ‘L’ denotes either the high or low intensity exercise session, respectively, from which the sample was collected; the numbers ‘01-14’, denotes the participant number.

4.3.1 Urinary pH and Osmolality

The mean urinary pH value of the samples prior to buffering was 6.12 ± 0.87 with a range of 4.99-8.40 (measured using a digital C830 pH meter; Consort, Belgium). The majority of these values were within the range reported in the medical literature of 4.80-7.50 (Stedman, 2000), with the exception of the four samples xH05, yH05, yH12 and yL12, which measured 7.56, 7.81, 7.90 and 8.30, respectively. It is possible that participants 05 and 12 may have experienced urinary alkalosis caused by excessive levels of bicarbonate excretion in the urine at the time of collection. Strong variations in urinary pH between samples can lead to ^{1}H NMR signal shifts (Beckonert *et al.*, 2007), hence the buffering step employed (Chapter 2). The data bucketing process employed, also known as binning, during spectral integration (Chapter 2) reduces the effect of pH-induced shifts, ensuring that the same ^{1}H NMR signals are integrated correctly across all samples with such variation (Craig *et al.*, 2006). The urinary pH and osmolality values for each participant are presented in Appendix 4.1 for descriptive purposes.

The mean osmolality value of the urine samples was 656 ± 378 with a range of 80-1727 mOsm/kg H_2O (measured using an Advanced Model 2020 Multi-sample Osmometer, Advanced Instruments; Norwood, Massachusetts USA). The majority of these values were within the range reported in the medical literature of 50-1400 mOsm/L (Stedman, 2000), with the exception of samples yH01, xL01 and xH08, which measured 1551, 1727 and 1637 mOsm/kg H_2O, respectively. Stedman (2000) reported the osmolality values as units of osmolarity (possibly by error); however, since 1 L of water weighs 1 kg, the range of values

in the medical text was considered as an appropriate reference comparable to the values measured in this study. Urine osmolality varies extensively in response to water consumption and therefore dehydration may also explain the high osmolality values in these three samples. Urine osmolality can increase to 1,200 mOsm/kg H_2O in the absence of water intake during overnight sleep and decrease rapidly to 50 mOsm/kg H_2O upon the consumption of water (Sands and Layton, 2009). Thus, the variability measured in urine osmolality values between participants in the present study (Appendix 4.1) may be the result of water intake in various quantities. Standardising water intake should be considered in prospective urine studies in an attempt to normalise osmolality values between participants, which may minimise concentration differences between urine samples.

4.3.2 PCA of ^{1}H NMR Spectral Data of Urine: Outlier Detection and Management

PCA was performed on the 600 MHz ^{1}H NMR spectra to investigate the effect of isokinetic exercise on urine metabolites without the influence of a predetermined classifier (unsupervised) and to detect potential outlying spectra, since outliers can mathematically skew data models and usually fail to provide information coherent with representative scores. The urinary dataset (excluding the dark regions TSP and HDO) was initially normalised to the sum of intensities to account for differences in urine volumes collected and concentrations between samples.

Figure 4.1A presents the PCA scores plot of the HIE dataset, whereby PC1 and PC2 explain 25.6% and 22.1% of variance, respectively. The scores xH10 and yH10 (participant 10) fell outside of Hotelling's 95% confidence limits (ellipses) and were located in the lower-right quadrant. The loadings plot presented in Figure 4.1B elucidated the spectral integrals in the lower-right quadrant as the variable weightings on these outlying scores. Consistent with the loadings, visual inspection of the spectra for xH10 and yH10 showed the presence of pronounced signals at δ 2.16, 2.18, 3.67, 3.90, 5.12, 7.14, 7.31, 7.36 and 7.45 (Figure 4.2), which were not present in the other spectra. As illustrated in Figure 4.2, these signals were assigned, according to the literature (Nicholls *et al.*, 1995; Spraul *et al.*, 2003), to the parace-

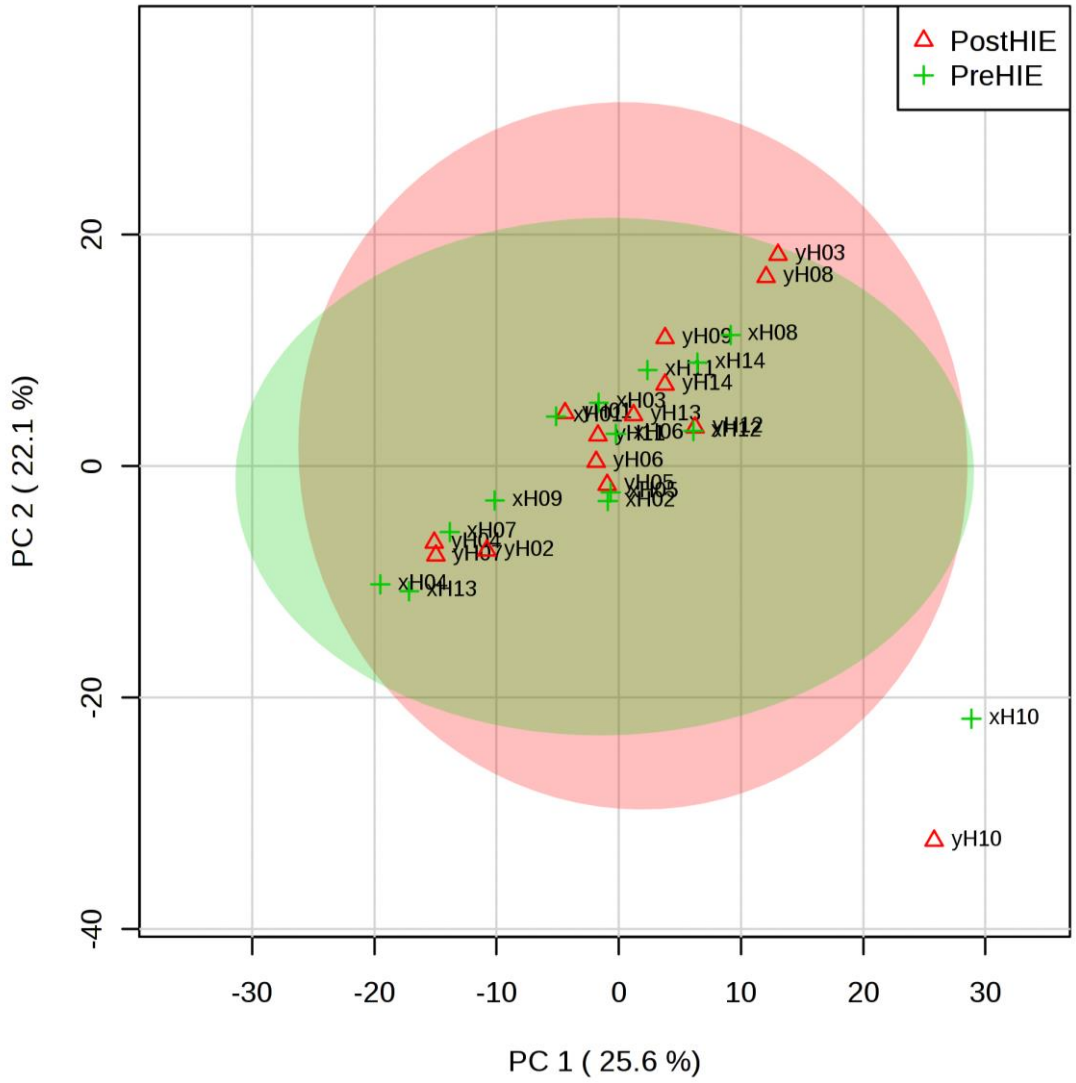


Figure 4.1A HIE Unsupervised Model: PCA scores plot of 600 MHz ^{1}H NMR spectra of urine, normalised to sum of intensities, taken from 14 healthy men (+) pre- and (Δ) post-HIE. PC1 and PC2 explain 25.6% and 22.1% of variance, respectively. The pre- and post-HIE scores for participant 10 fell outside of Hotelling's 95% confidence ellipses. Throughout this chapter, the green and red ellipses in all data models denote the 95% confidence regions for the pre- and post-exercise scores, respectively.

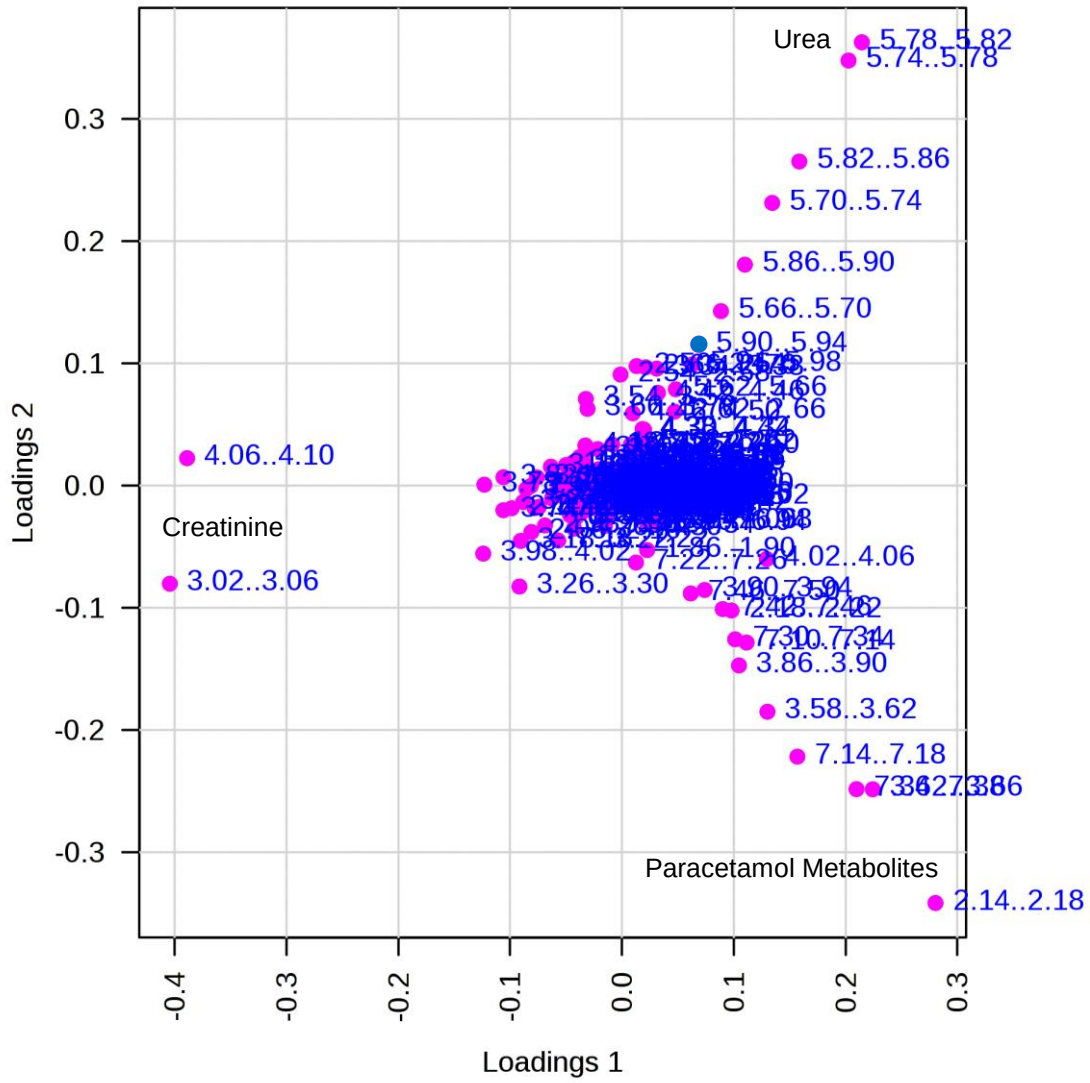


Figure 4.1B HIE Unsupervised Model: PCA loadings plot showing the weighting of variables (spectral integrals) on the HIE scores, which were assigned to creatinine and urea, metabolites naturally abundant in urine spectra, and paracetamol metabolites (glucuronide and sulphate conjugates).

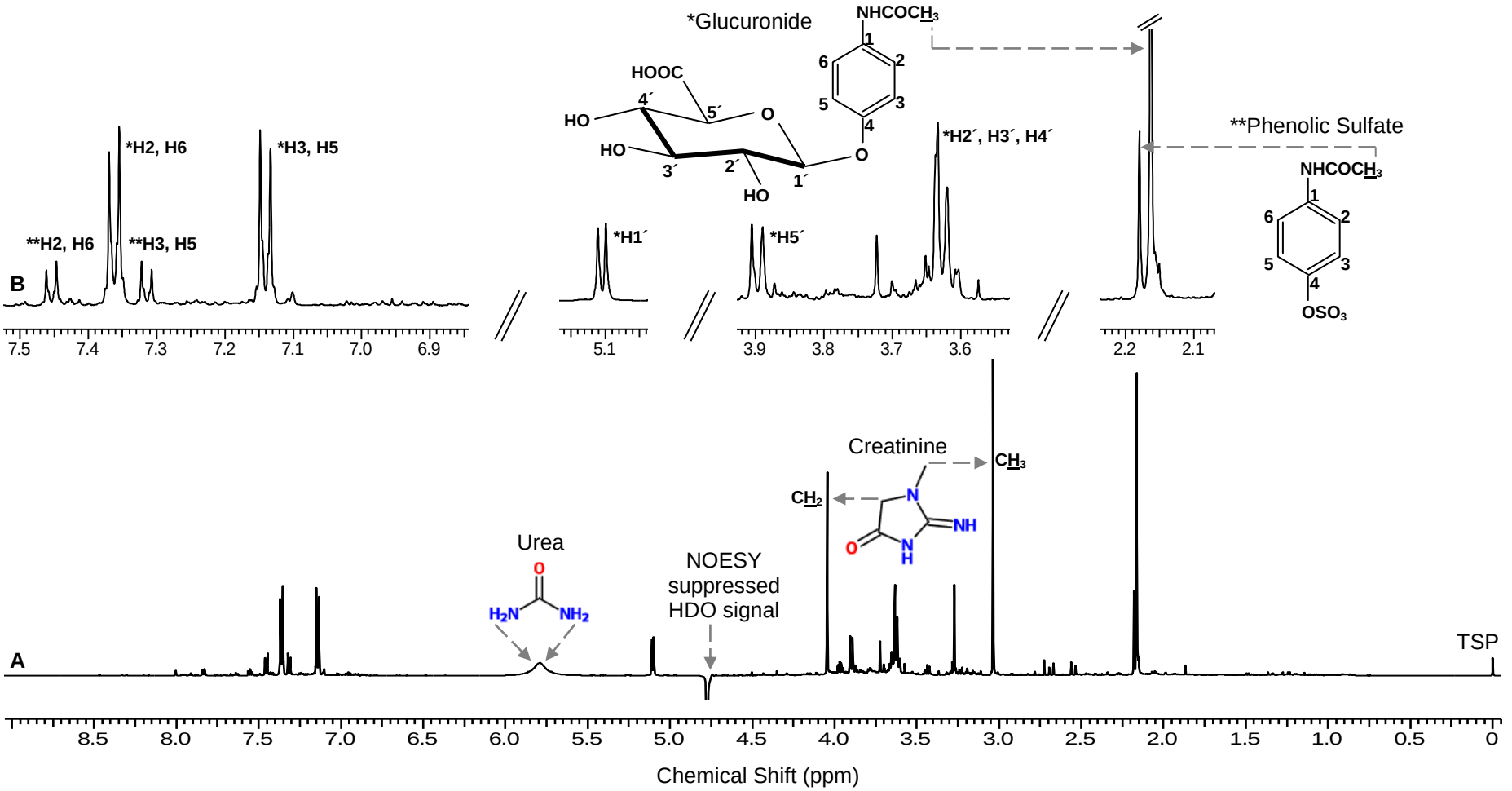


Figure 4.2 (A) 600 MHz ^{1}H NMR spectrum (256 scans) of urine taken from participant 10 (yH10); signals were assigned to creatinine and urea (metabolites naturally abundant in urine) with reference to the molecular structures. **(B)** Selected expanded regions showing signals assigned to the paracetamol metabolites glucuronide and phenolic sulphate with reference to the molecular structures.

tamol metabolites glucuronide (δ 2.16, 3.67, 3.90, 5.12, 7.14 and 7.36) and phenolic sulfate (δ 2.18, 7.31 and 7.45), together with the metabolites creatinine (δ 3.05 and 4.06) and urea (δ 5.80) which were naturally abundant in all urine spectra.

Unknown to the author at the time of biofluid collection and exercise participation, it is possible that participant 10 violated one of the inclusion criteria by ingesting paracetamol prior to participation. To resolve the issue of the outlying scores, two options were explored: deletion of integrals (variables) corresponding to the exogenous signals of paracetamol metabolites from the entire dataset; deletion of the outlying scores (samples) xH10 and yH10 to exclude participant 10.

Henceforth, resonance assignment was carried out based on chemical shift values and signal multiplicity in relation to molecular structures according to the literature (Holmes *et al.*, 1997; Pechlivanis *et al.*, 2010; Bouatra *et al.*, 2013), and the Human Metabolome Database (www.hmdb.ca) and the Urine Metabolome Database (www.urinemetabolome.ca).

To explore the option of variable editing, the spectral integrals corresponding to the signals of paracetamol metabolites (δ 2.16, 2.18, 3.67, 3.90, 5.12, 7.14, 7.31, 7.36 and 7.45) were deleted from the entire spectral dataset and PCA was reapplied. The resulting PCA scores plot is presented in Figure 4.3A, whereby PC1 and PC2 explains 31.3% and 16.4% of variance, respectively. As a result, the original outlying scores xH10 and yH10 fell within Hotelling's 95% confidence ellipses and yH11 fell outside of the ellipses across PC2. The loadings plot presented in Figure 4.3B illustrates that the spectral integrals 1.30-1.34, 1.34-1.38 and 4.10-4.14 were the variable weightings on the score yH11. Consistent with the loadings, visual inspection of the spectra for participant 11 (Appendix 4.2) showed pronounced signals following exercise, in comparison to other individuals, assigned to the CH_3 and CH protons of lactate at δ 1.33 and 4.13, respectively, which indicates a rapid lactate clearance from the blood. The urinary pH and osmolality values of sample yH11 was pH 7.32 and 762 mOsm/kg H2O, respectively, which were within the normal published range

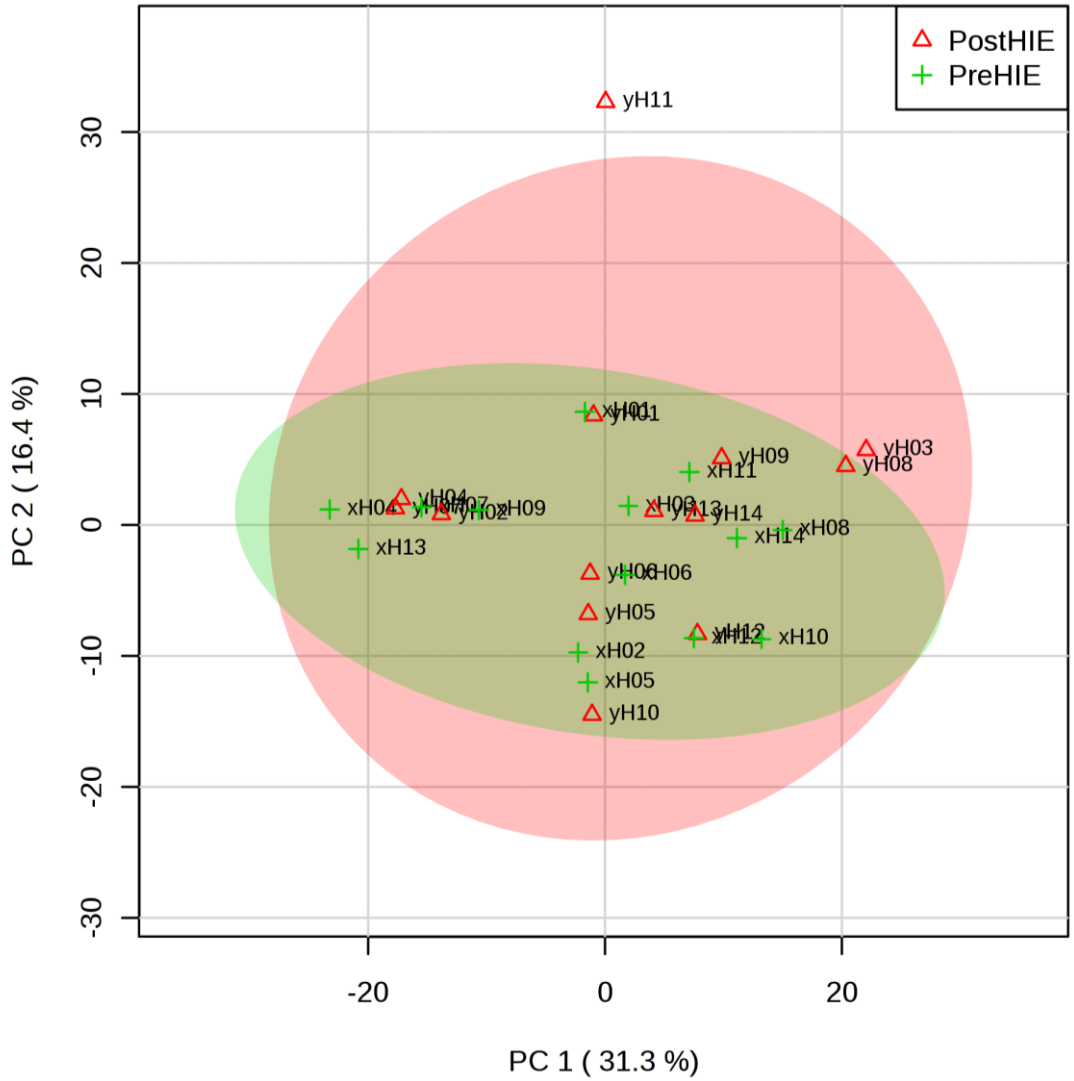


Figure 4.3A Refined HIE Unsupervised Model (1): PCA scores plot of 600 MHz ^{1}H NMR spectra of urine, normalised to sum of intensities, taken from 14 healthy men (+) pre- and (Δ) post-HIE. PC1 and PC2 explain 31.3% and 16.4% of variance, respectively. The integrals of paracetamol metabolites were deleted from all spectral data. The original outliers xH10 and yH10 fell within Hotelling's 95% confidence ellipses, whereas, yH11 fell outside of the ellipses.

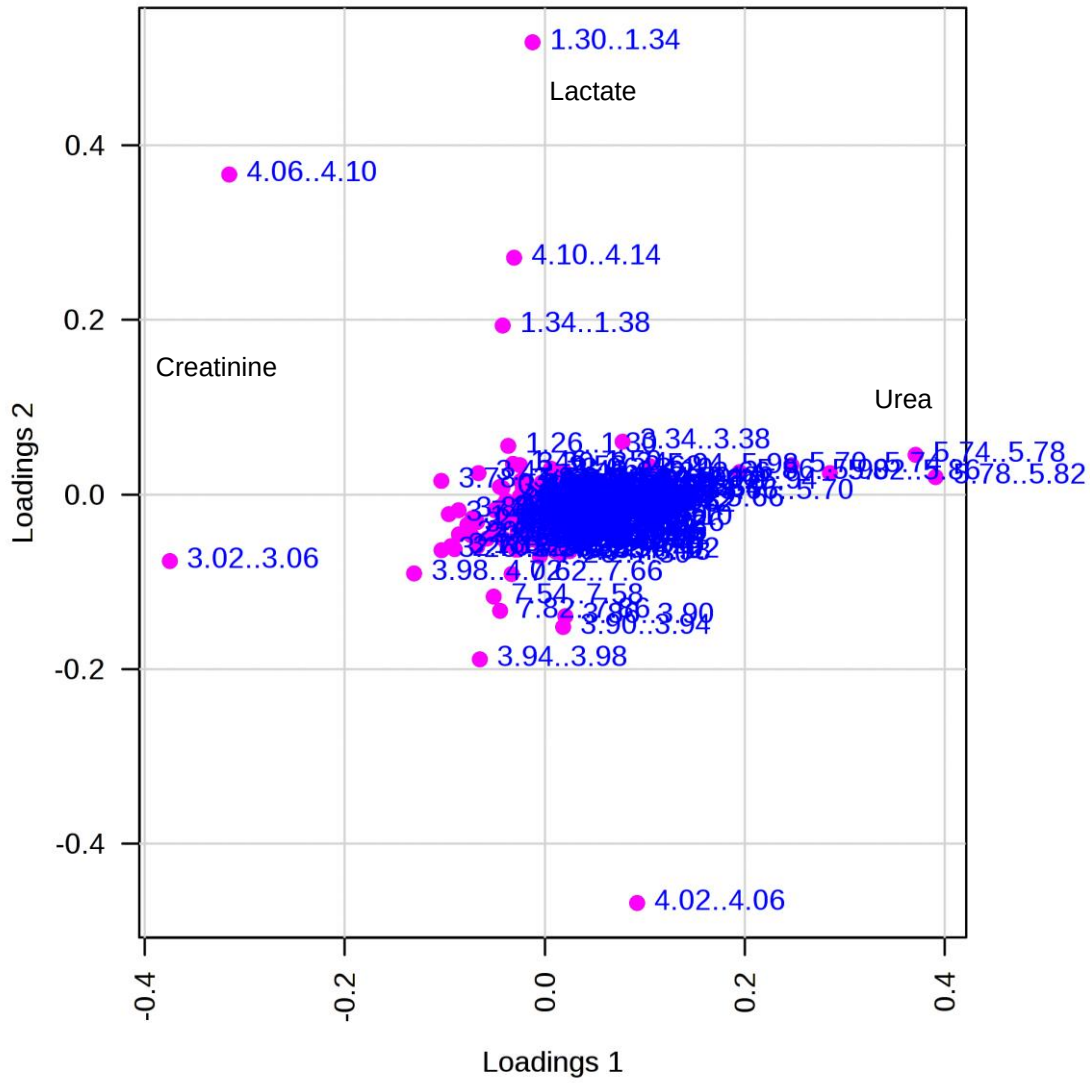


Figure 4.3B Refined Unsupervised Model (1): PCA loadings plot showing the weighting of variables (spectral integrals) on the HIE scores, after the deletion of paracetamol metabolites (δ 2.16, 2.18, 3.67, 3.90, 5.12, 7.14, 7.31, 7.36 and 7.45). The integrals were assigned to creatinine (δ 3.05 and δ 4.06), urea (δ 5.80) and lactate (δ 1.33 and δ 4.13).

and no other unusual peaks were present in this spectrum; thus, yH11 was not deleted from the model.

To explore the option of sample editing, the outlying scores xH10 and yH10 were deleted from the spectral dataset and PCA was reapplied. The resulting PCA model presented in Figures 4.4A and 4.4B exhibited the same outcome as the variable editing method in terms of percentage of variance explained across PC1 and PC2, score separation patterns and spectral integrals elucidated in the loadings. Thus, variable editing on the dataset in this study permitted the inclusion of participant 10 in the HIE session thus retaining the original number of participants and reshaped the scores to include the former outliers within Hotelling's 95% confidence ellipses in the revised model. However, employing the variable editing method to manage outlying scores is only applicable in the context in which it is used and thus, other scenarios may require sample deletion (e.g. analytical outliers).

The PCA scores plot of the LIE dataset is presented in Figure 4.5A, whereby PC1 and PC2 explain 31.2% and 15.1% of variance, respectively. All scores fell within Hotelling's 95% confidence ellipses, hence no outliers were detected, and the scores separated according to inter-individual variation and effect of LIE. The loadings plot presented in Figure 4.5B elucidated the same variable weightings as the HIE model (Figure 4.3B).

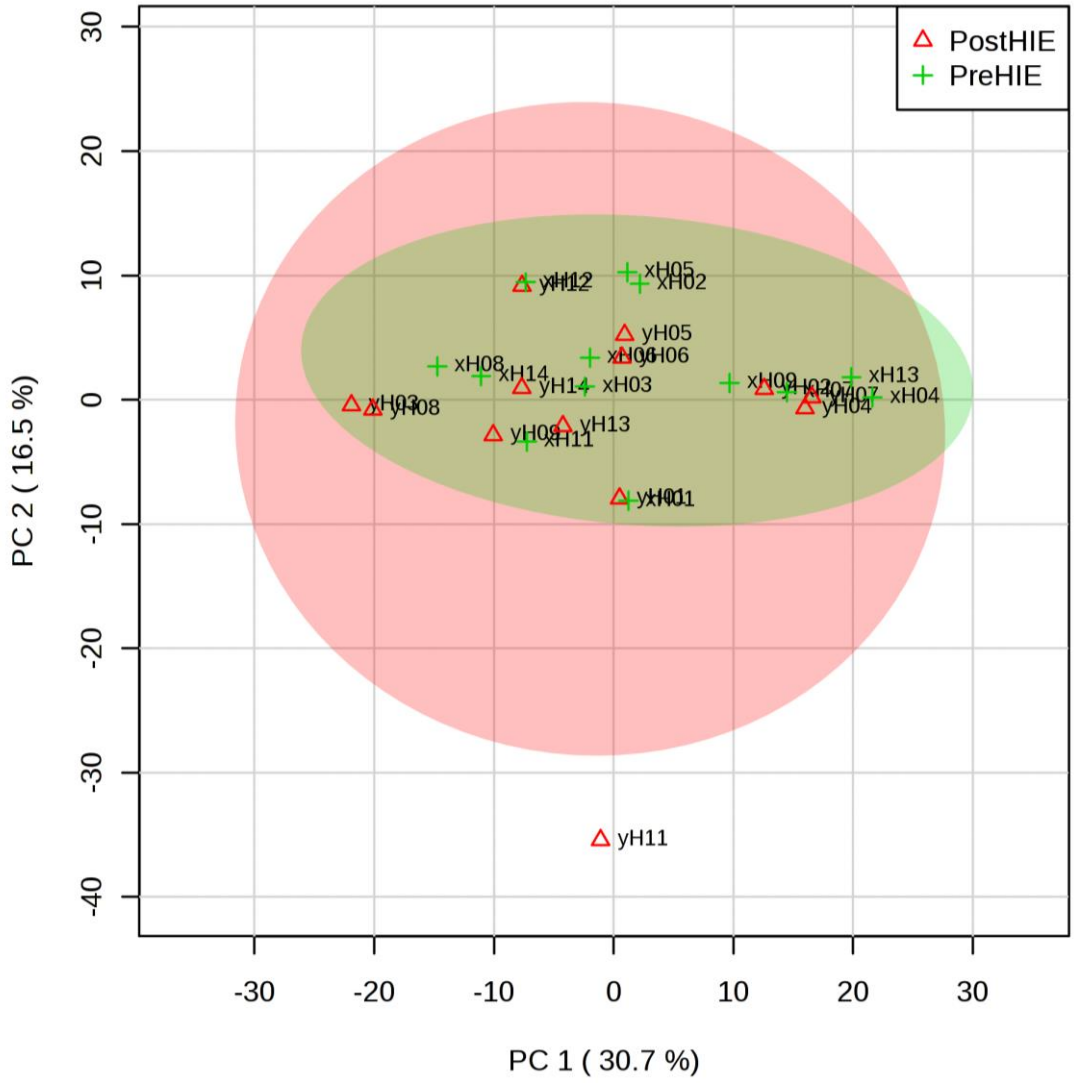


Figure 4.4A Refined HIE Unsupervised Model (2): PCA scores plot of 600 MHz ^{1}H NMR spectra of urine, normalised to sum of intensities, taken from 13 healthy men (+) pre- and (Δ) post-HIE. PC1 and PC2 explain 30.7% and 16.5% of variance, respectively. The original outlying scores xH10 and yH10 were deleted from the data. The score yH11 fell outside of Hotelling's 95% confidence ellipses.

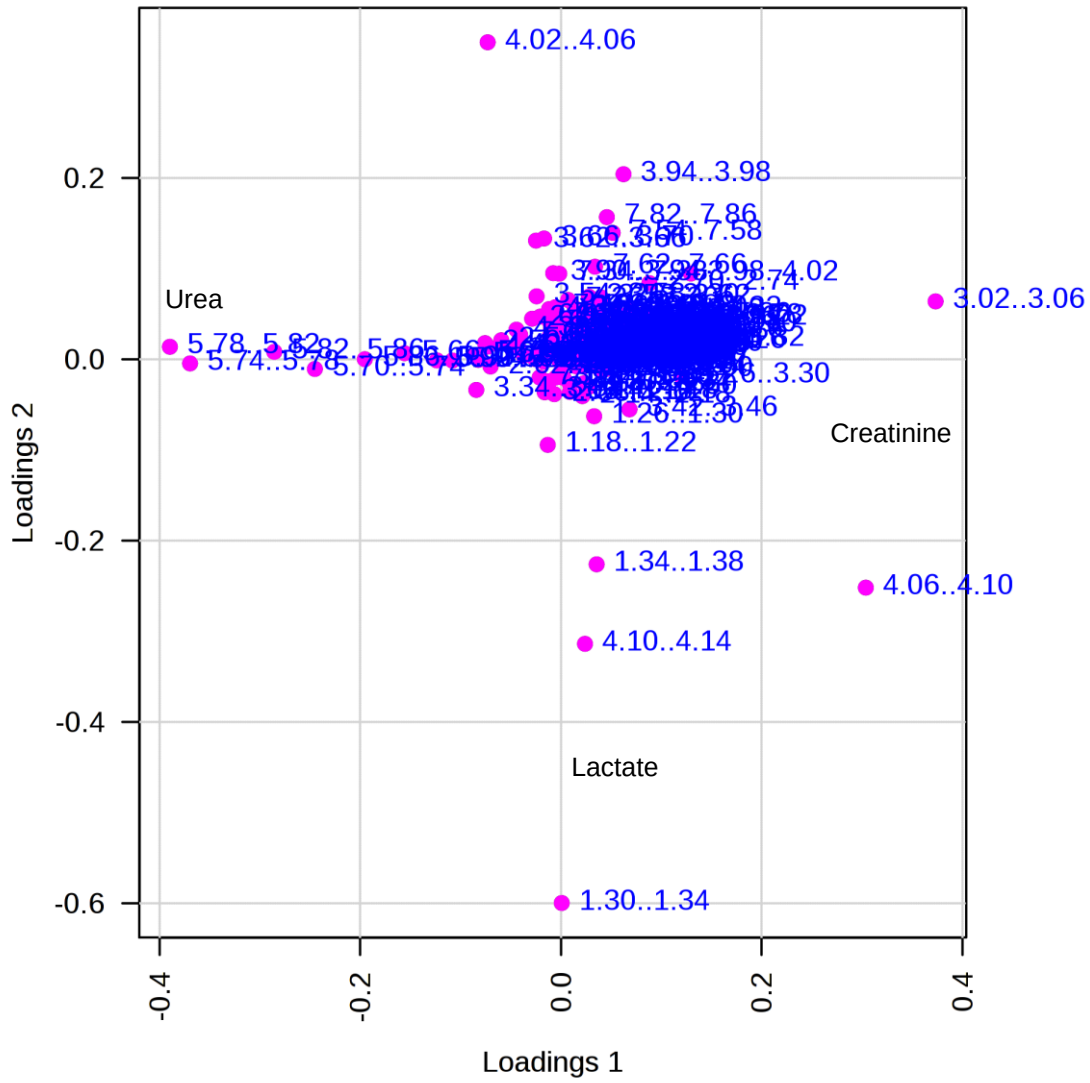


Figure 4.4B Refined HIE Unsupervised Model (2): PCA loadings plot showing the weighting of variables (spectral integrals) on the HIE scores, after the deletion of the outlying scores (xH10 and yH10) elucidated in the original model. The integrals were assigned to creatinine (δ 3.05 and δ 4.06), urea (δ 5.80) and lactate (δ 1.33 and δ 4.13).

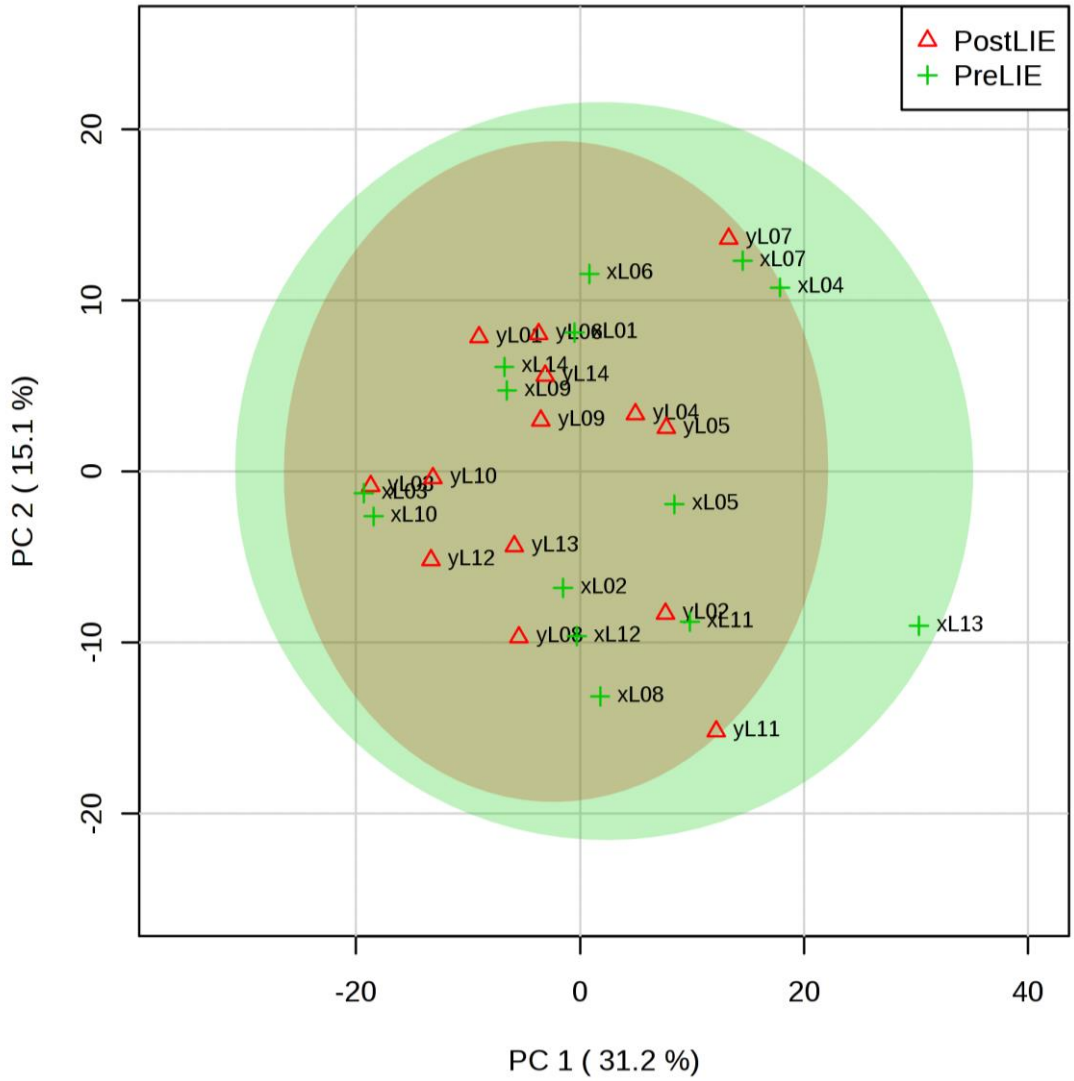


Figure 4.5A LIE Unsupervised Model: PCA scores plot of 600 MHz ^{1}H NMR spectra of urine, normalised to sum of intensities, taken from 14 healthy men (+) pre- and (Δ) post-LIE. PC1 and PC2 explain 31.2% and 15.1% of variance, respectively. All scores fell within Hotelling's 95% confidence ellipses.

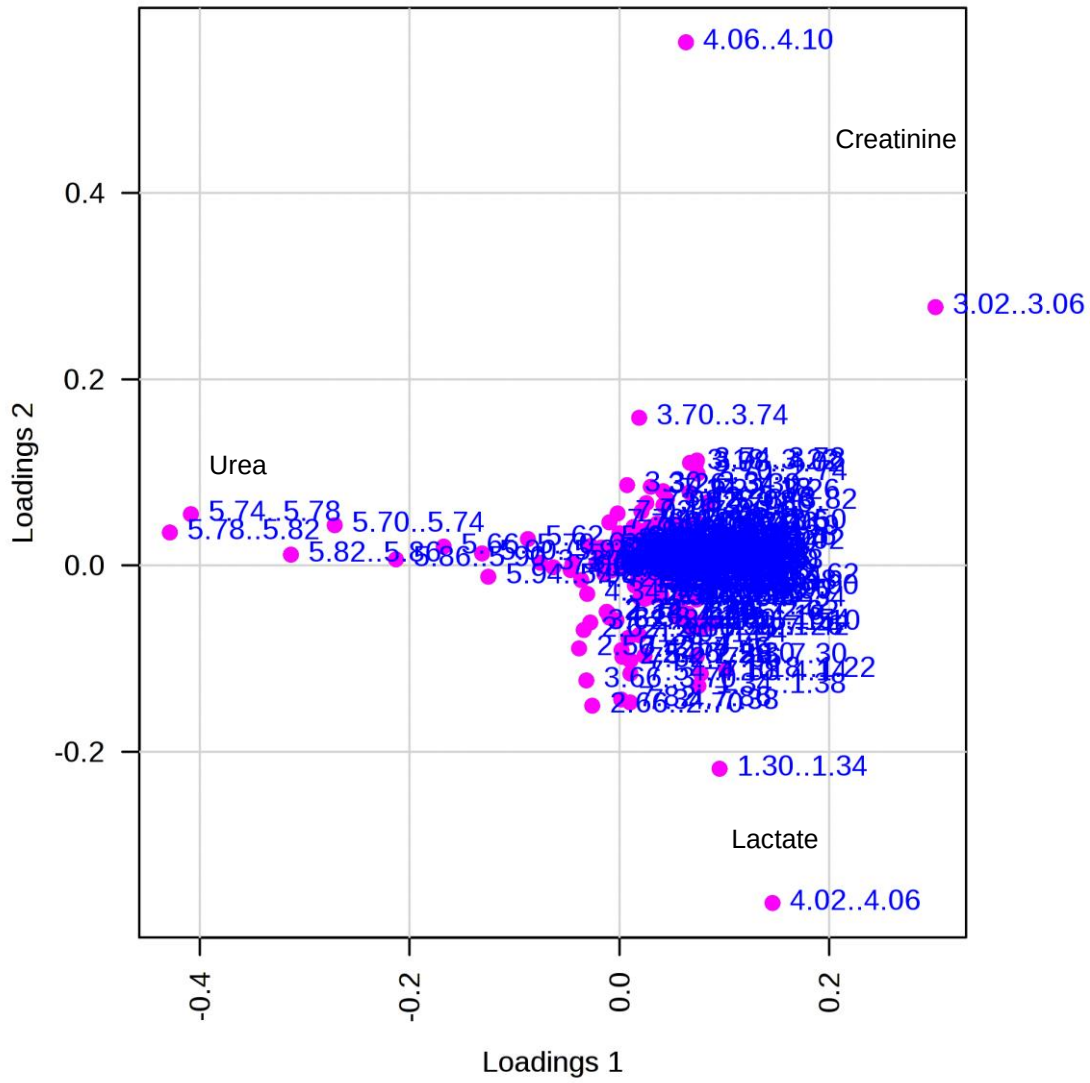


Figure 4.5B LIE Unsupervised Model: PCA loadings plot showing weighting of variables (spectral integrals) on the LIE scores, which were assigned to creatinine (δ 3.05 and δ 4.06), urea (δ 5.80) and lactate (δ 1.33).

Urinary creatinine, urea and lactate were the dominant variable weightings on the PCA models of the HIE and LIE datasets, normalised to sum of intensities. Enea *et al.* (2010) reported the same finding and therefore deleted the integrals of urinary creatinine, urea and lactate from the entire dataset and then reapplied PCA to investigate the effect of cycling exercise on other metabolites. Whereas, Pechlivanis *et al.* (2010) deleted the urinary urea integrals prior to performing PCA and reported that normalising to the creatinine signal at δ 3.05 provided a more robust model in terms of component vector values (e.g. variance explained) and overcame the influence of the intense lactate signals. This research group then deleted the lactate integrals to further investigate the effect of sprint running on other metabolites. Therefore in the present study, univariate statistical analysis was performed on the spectral integrals of creatinine, urea and lactate to determine whether or not HIE and LIE had an effect, prior to further spectral data editing.

4.3.3 Univariate Statistical Analysis of Urinary Creatinine, Urea and Lactate Levels

Since the integrated spectral datasets provided numerical values of the area under the peaks of ^{1}H NMR signals, the integrals of creatinine (δ 3.05), urea (δ 5.80) and lactate (δ 1.33) were subjected to univariate statistical analysis after normalisation. Initially, creatinine, urea and lactate integrals were normalised to the TSP (δ 0.00) integral to account for any analytical differences between samples. Lastly, the original integrals of urea and lactate were normalised to creatinine to account for differences in glomerular filtration rates between participants (Waikar *et al.*, 2010).

No significant differences were found in urinary creatinine, urea and lactate levels normalised to TSP, or urea and lactate levels normalised to creatinine, between pre- and post-HIE and LIE (Table 4.1), suggesting that isokinetic exercise did not affect urinary excretion of these metabolites at either the 80%MVC or 40%MVC intensities. Since inter-individual variation was greater than the effect of exercise, no uniform change was exhibited in the values of urinary creatinine, urea and lactate amongst the 14 participants following isokinetic exercise and thus, the SD was greater than the mean in several of the values.

Table 4.1 Results from univariate statistical analysis of the semi-quantitative values (mean ± SD) of urinary creatinine, urea and lactate levels sampled and measured pre- and post-HIE and LIE.

	HIE			LIE		
Metabolite	**Pre**	**Post**	***p* value**	**Pre**	**Post**	***p* value**
Creatinine/TSP	28.26 ± 23.78	54.02 ± 92.09	0.265[a]	18.85 ± 13.47	27.69 ± 44.58	0.465[a]
Urea/TSP	44.75 ± 49.40	101.37 ± 217.03	0.875[b]	26.97 ± 16.77	69.54 ± 144.32	0.594[b]
Lactate/TSP	0.87 ± 1.30	4.98 ± 8.45	0.074[b]	0.60 ± 0.60	4.0 ± 10.50	0.245[b]
Urea/Creatinine	1.69 ± 0.83	1.91 ± 0.83	0.385[a]	2.01 ± 1.08	2.43 ± 0.93	0.134[a]
Lactate/Creatinine	0.03 ± 0.01	0.19 ± 0.48	0.248[a]	0.03 ± 0.02	0.14 ± 0.33	0.272[b]

p values as determined by either the Pared *t*-Test[a] or Wilcoxon Signed Rank Test[b], following Normality Distribution Tests (Appendix 2.6)

Creatinine is excreted in the urine in concentrations ranging between 2.21-44.2 mM (Stedman, 2000) and these quantities are influenced by both the glomerular filtration rate and muscle mass, and therefore varies according to renal function, athletic status (Enea *et al.*, 2010; Mukherjee *et al.*, 2014), age and gender (Slupsky *et al.*, 2007). Creatinine measurements serves several roles including a biomarker of creatine metabolism (Wyss and Kaddurah-Daouk, 2000), a variable in a calculation to estimate glomerular filtration rate in relation to kidney health (Waikar *et al.*, 2010; Endre *et al.*, 2011), and a normalisation variable for chemometric analysis in urinary metabolomics to profile exercise-induced metabolic stress (Pechlivanis *et al.*, 2010; Mukherjee *et al.*, 2014; Daskalaki *et al.*, 2015). Urinary creatinine gives rise to intense singlet signals at δ 3.05 and 4.06 in the spectrum assignable to the CH_3 and CH_2 protons, respectively (Figure 4.2).

In the present study, levels of urinary creatinine were highly correlated with lactate (Spearman's rho, r=0.852, p=0.001) and urea (Spearman's rho, r=0.854, p=0.001), suggesting that inter-individual variability in lactate and urea levels was the result of individual differences in glomerular filtration rates and muscle mass. The non-significant findings in urinary urea and lactate levels, normalised to creatinine, between pre- and post-exercise samples suggest that muscular stress imposed by the HIE and LIE interventions did not alter glomerular filtration rates and thus, interventions of this nature may be of a clinical advantage to patients with renal injury or disease undergoing physiotherapy. However, this study was performed on 14 healthy individuals and it is unlikely that clinical populations will exhibit the same response to concentric isokinetic exercise in terms of urinary lactate and urea formation, and alterations in glomerular filtration rates.

Since no significant change has been reported in urinary creatinine ^{1}H NMR profiles after exercise-induced metabolic stress (Enea *et al.*, 2010; Pechlivanis *et al.*, 2010; Mukherjee *et al.*, 2014) or muscular stress (results of this study), the spectral data was normalised to creatinine (δ 3.05) in further multivariate analysis by chemometrics to account for individual differences in glomerular filtration rates (Section 4.3.4). The limitation of data

normalisation to the creatinine signal is that other urinary metabolite integral values may be overestimated or underestimated (Waikar *et al.*, 2010), which has implications in terms of potential clinical misdiagnosis and exaggerated values in research data, particular because creatinine excretion is dependent on muscle mass. If the creatinine concentration is unusually high or low for an individual compared to other analytes in a given dataset, then the above implications should be recognised by the clinician or researcher prior to interpretation.

Urea is an end product of protein catabolism and amino acid degradation, and is formed in the liver, kidneys and muscles via deamination of branched-chain amino acids (BCAA) during the process of ornithine formation from arginine when ammonia enters the urea cycle (Viru and Viru, 2001), as illustrated in Figure 4.6. Urea is naturally abundant in the ^{1}H NMR spectrum of urine and is excreted in concentrations ranging between 0.1-1.0 M (Stedman, 2000). The findings of this study revealed no significant change in urinary urea excretion following concentric isokinetic exercise and thus, this intervention may be appropriate for populations with fatigue syndrome since it appears to attenuate a catabolic state.

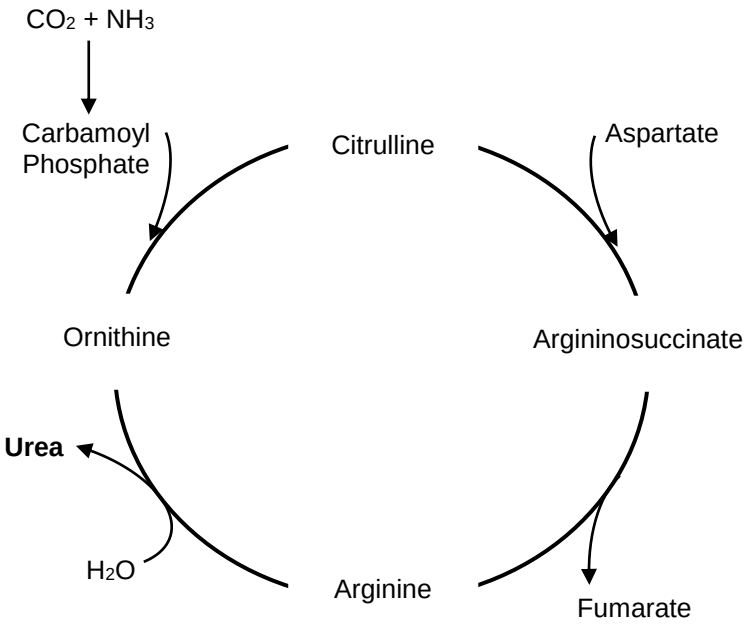


Figure 4.6 Urea Cycle.

The blood content of lactate is an established biomarker of anaerobic metabolism indicative of exercise intensity and tolerance, and is excreted in the urine in concentrations ranging between 0.55-13.3 mM (Stedman, 2000). ^{1}H NMR spectroscopy studies on urine have reported marked increases in lactate excretion after exercise-induced metabolic stress from cycling (Enea *et al.*, 2010; Mukherjee *et al.*, 2014) and running (Pechlivanis *et al.*, 2010), or a combination of the two (Daskalaki *et al.*, 2015). The findings of the present study revealed no significant change in urinary excretion of lactate following concentric isokinetic exercise.

Since no significant change was found in urinary creatinine, urea and lactate levels following muscular stress imposed by the HIE and LIE interventions, lactate and urea integrals were deleted from the entire spectral dataset for further multivariate statistical analysis by chemometrics. Two options were explored for the creatinine integrals as reported below.

4.3.4 Effect of Spectral Data Normalisation on Chemometric Models

Two separate data normalisation techniques were employed in chemometric analysis to optimise the variance explained by PC1 and PC2, to provide the most robust models possible: Data normalisation to the creatinine signal at δ 3.05, to account for individual differences in glomerular filtration rates; deletion of the creatinine integrals and data normalisation to sum of intensities, to account for differences in sample concentrations.

The results presented in Table 4.2 shows that data normalisation to creatinine provides the most robust model in terms of percentage of variance explained across PC1 and total variance explained. Thus henceforth in this chapter, all multivariate statistical analysis are based on normalisation to creatinine.

Table 4.2 Statistical models generated from the urinary spectral dataset and variance explained

	% Variance Explained		
PCA Model (Appendix 4.3)	**PC1**	**PC2**	**Total**
HIE, normalised to creatinine	28.8	15.5	44.3
HIE, normalised to sum of intensities	17.9	15.0	32.9
LIE, normalised to creatinine	23.3	17.1	40.4
LIE, normalised to sum of intensities	19.7	18.6	38.3

4.3.5 The Effect of HIE and LIE on Urinary Metabolites after Model Refinement

PLS-DA was performed on the 600 MHz ^{1}H NMR spectra of urine to discriminate between classes with exercise as the classifier (supervised) and to elucidate spectral integrals associated with the classifier to inform resonance assignment, after the deletion of the pronounced signals of lactate (δ 1.33 and δ 4.13) and urea (δ 5.80). The creatinine signal at δ 3.05 was included as an internal standard for normalisation as reported previously (Section 4.3.4), whereas the creatinine signal at δ 4.06 was excluded.

Figure 4.7A presents the PLS-DA scores plot of the HIE dataset, whereby component 1 and 2 explain 8.8% and 23.5% of variance, respectively. A partial class distinction was achieved since the majority of scores separated across component 1 according to the classifier (pre- and post-HIE), with an overlap of scores (xH01, yH07, xH12 and xH14), whereas separation across component 2 was mainly attributable to inter-individual variation. Participants 07, 12 and 14 exhibited no separation between pre- and post-scores indicating no modifications in urinary metabolites in response to HIE. The PLS-DA loadings plot presented in Figure 4.7B illustrates that the signals of hippurate (δ 3.96, δ 7.54, δ 7.64 and δ 7.84), glycine (δ 3.57), citrate (δ 2.54 and δ 2.69) and trimethylamine *N*-

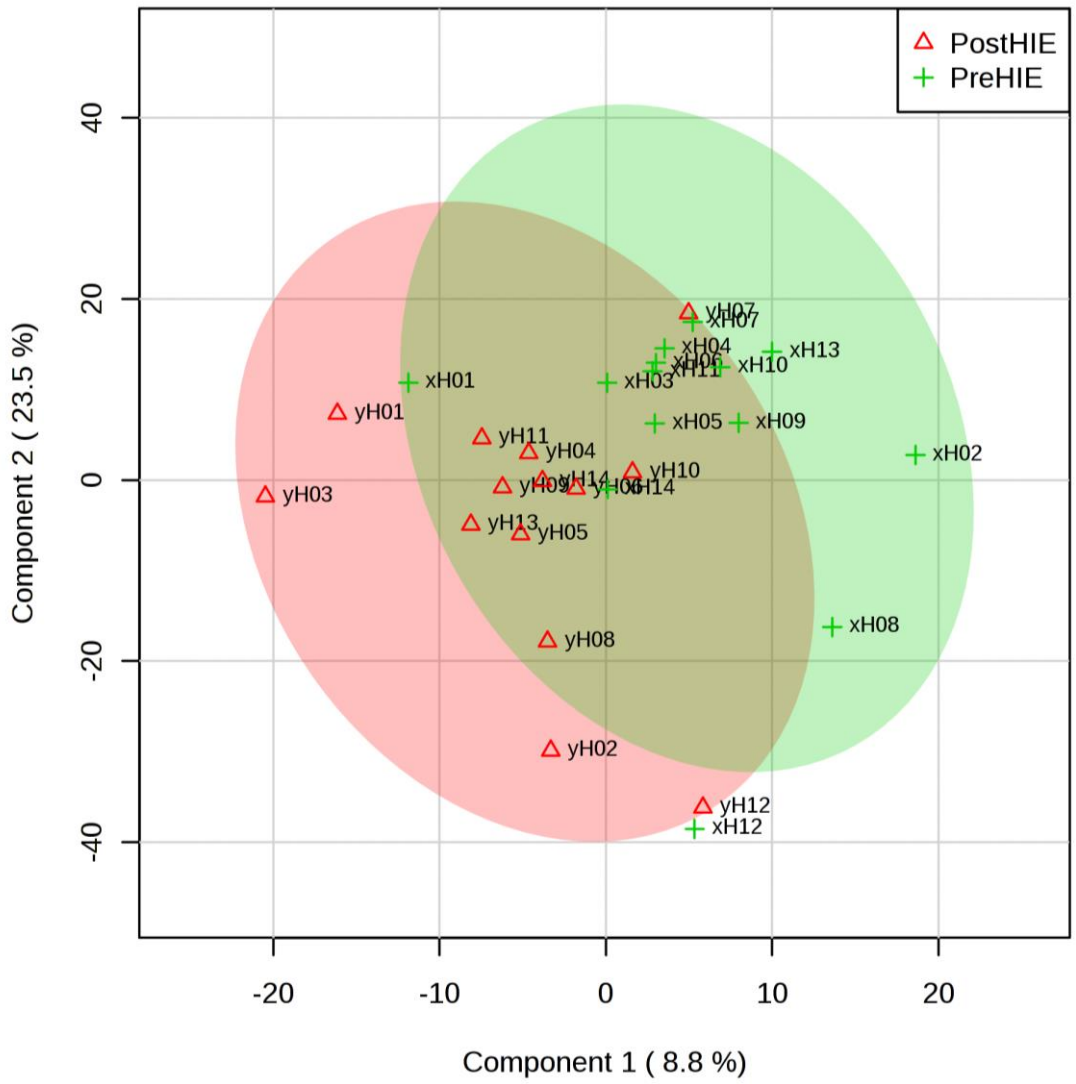


Figure 4.7A HIE Supervised Model: PLS-DA scores plot of 600 MHz ^{1}H NMR spectra of urine (excluding lactate, creatinine and urea) normalised to the creatinine signal (δ 3.05), taken from 14 healthy men (+) pre- and (Δ) post-HIE. Component 1 and 2 explain 8.8% and 23.5% of variance, respectively. Scores mostly separated according to class (effect of HIE) for all participants, except participants 07, 12 and 14. A partial class distinction was established due score overlap between class clusters.

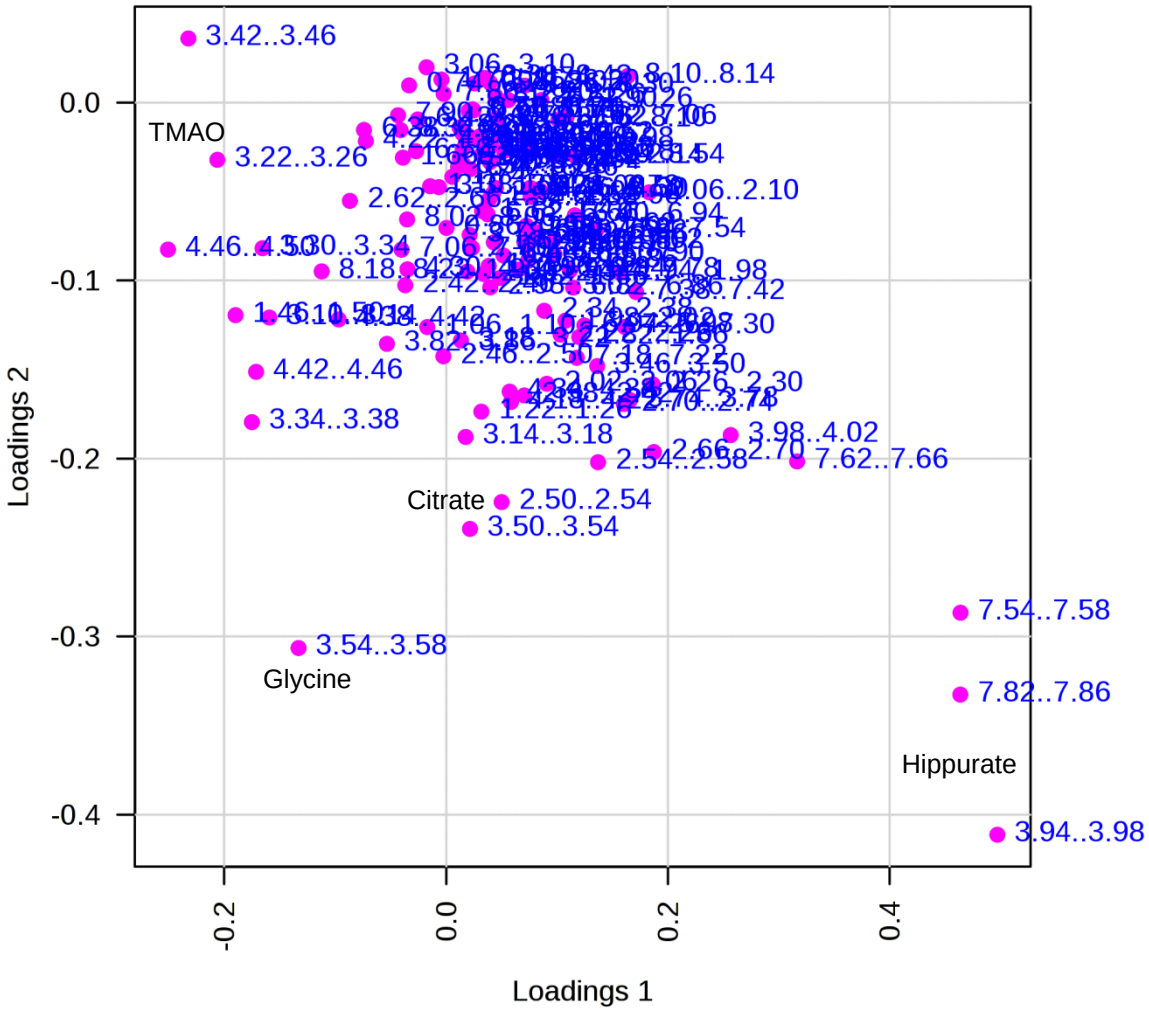


Figure 4.7B HIE Supervised Model: PLS-DA loadings plot showing the weighting of variables (spectral integrals) on the HIE scores, excluding lactate, creatinine and urea. The variables were assigned to the biomolecular markers of renal stress including hippurate (δ 3.96, δ 7.84, δ 7.54 and δ 7.64), glycine (δ 3.57), citrate (δ 2.54 and δ 2.69) and trimethylamine N-oxide (TMAO) (δ 3.27).

oxide (δ 3.27) were the strongest variable weightings associated with the separation of scores according to the classifier (effect of HIE).

The PLS-DA scores plot of the LIE dataset is presented in Figure 4.8A, whereby component 1 and 2 explains 11.9% and 15.9% of variance, respectively. The LIE data model also achieved a partial class distinction with an overlap of scores (yL02, yL07 and yL08). Participant 07 exhibited practically no separation in response to LIE, whereas participant 14 exhibited less separation compared to other individuals in the LIE model, indicating no modifications in urinary metabolites in response to LIE in these individuals, which is consistent with the HIE data model. The corresponding loadings plot presented in Figure 4.8B also elucidated the spectral variables hippurate, citrate glycine and trimethylamine *N*-oxide as the strongest variable weightings.

Since it appears that participants 07, 12 and 14 were not systemically perturbed by concentric isokinetic exercise, it is possible that these individuals were either accustomed to muscular stress or possess a reduced renal functional capacity to remove blood metabolites derived from exercise metabolism. In the case of the latter, there may be a mechanism involved or stressed, independent of the glomerular filtration rate since the dataset was normalised to creatinine. The urinary pH of participant 12 was slightly alkaline on both exercise sessions (pH 7.81 and 7.56) which may be an indication of kidney alkalosis; however, participants 07 and 14 fell within the values published in the medical literature (Stedman, 2000).

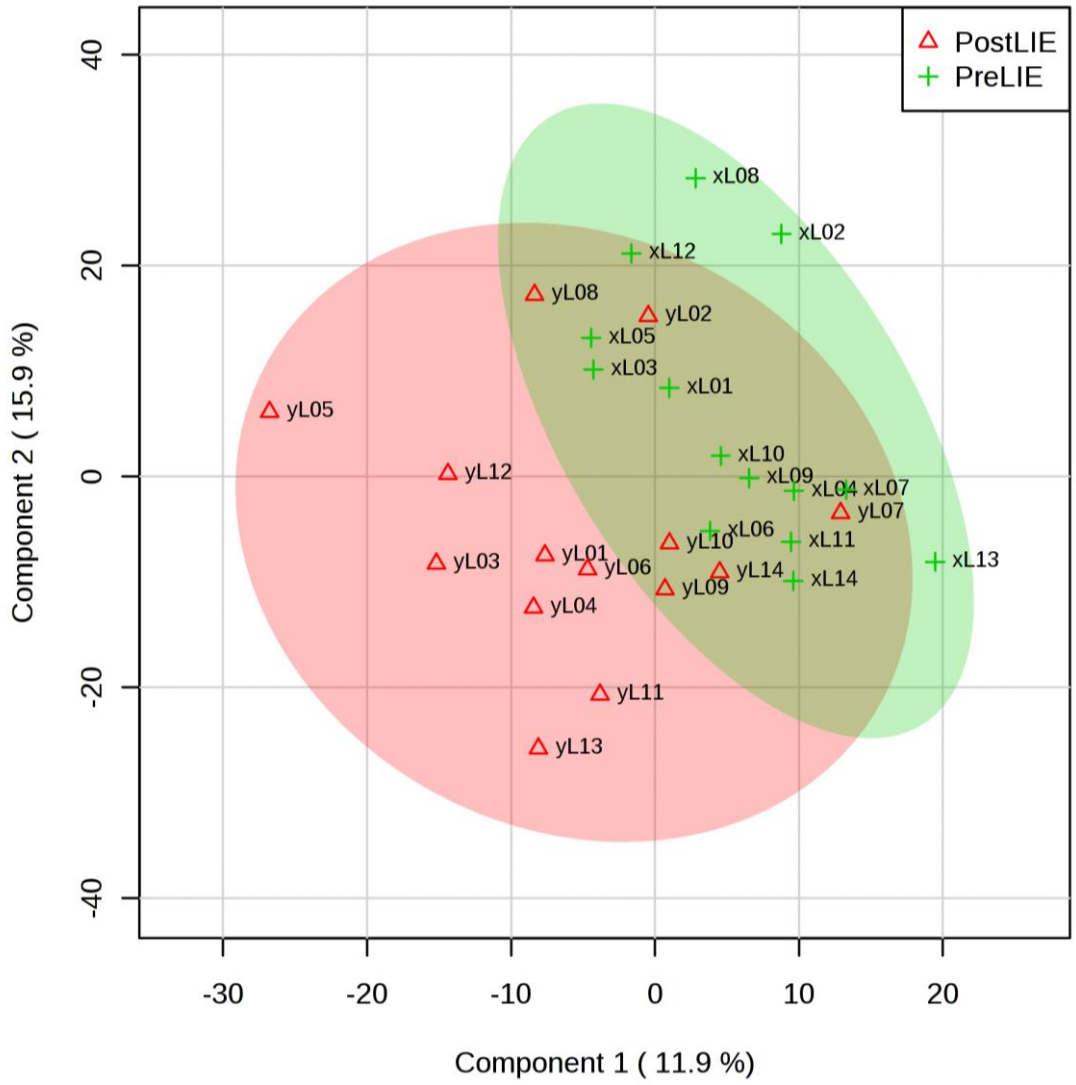


Figure 4.8A LIE Supervised Model: PLS-DA scores plot of 600 MHz ^{1}H NMR spectra of urine (excluding lactate, creatinine and urea), normalised to the creatinine signal (δ 3.05), taken from 14 healthy men (+) pre- and (Δ) post-LIE. Component 1 and 2 explain 11.9% and 15.9% of variance, respectively. Scores mostly separated according to class (effect of LIE) for all participants, except participant 07. A partial class distinction was established due to score overlap between class clusters.

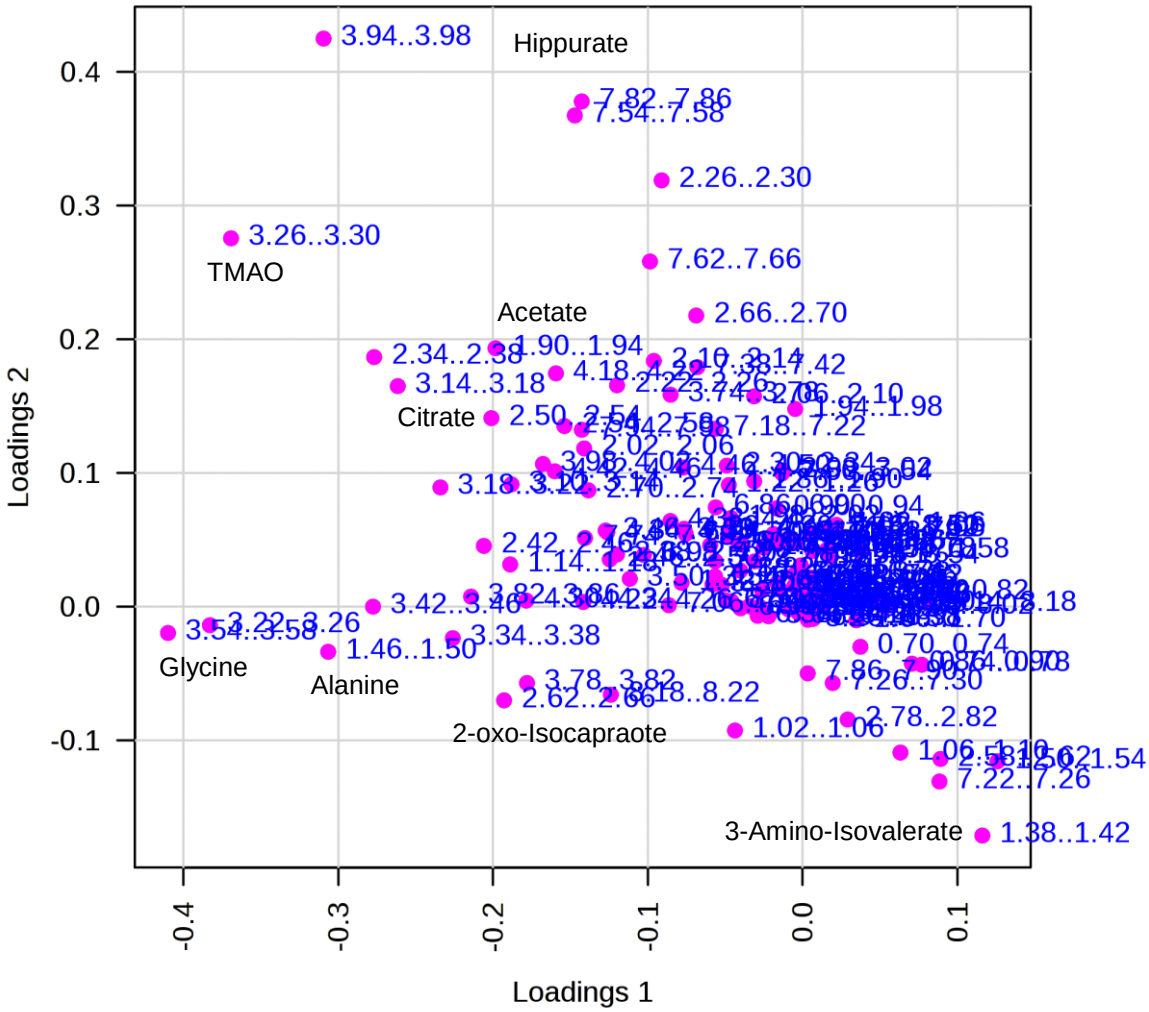


Figure 4.8B LIE Supervised Model: PLS-DA loadings plot showing the weighting of variables (spectral integrals) on the LIE scores, excluding lactate, creatinine and urea. The variables were assigned to the biomolecular markers of renal stress including hippurate (δ 3.96, δ 7.84, δ 7.54 and δ 7.64), glycine (δ 3.57), citrate (δ 2.54 and δ 2.69) and trimethylamine N-oxide (TMAO) (δ 3.27). Other integrals were assigned to anions of short-chain organic acids naturally excreted in the urine.

Figure 4.9 presents illustrative spectra obtained pre- and post-HIE from one participant to illustrate resonance assignment of metabolites strongly associated with the PLS-DA classifier (effect of HIE and LIE). Figure 4.9 shows a visible decrease in hippurate (3.67-fold), citrate (1.67-fold) and glycine (2.13-fold) signals post-exercise, and an increase in trimethylamine *N*-oxide (1.11-fold) was also exhibited however, the signal was truncated in Figure 4.9 due its pronounced intensity. Although these metabolites are naturally excreted in the urine, this specific change has previously been associated with acute renal injury related to rhabdomyolysis (Bairaktari *et al.*, 2002) and potential, indirect renal stress imposed by sprint running exercise (Pechlivanis *et al.*, 2010). The results of the present study suggest that concentric muscular loading imposed by isokinetic exercise of the knee extensor and flexors at 80%MVC and 40%MVC may modify metabolites associated with acute renal dysfunction, possibly due to the renal filtration of myoglobin released from stressed muscle into the blood stream (Speranza *et al.*, 2007). However, to verify that the HIE and LIE interventions caused an increase in the renal filtration of myoglobin, myoglobin concentrations in the blood and urine would require measurement by enzyme-linked immunoabsorbant assay.

Excessive amounts of trimethylamine *N*-oxide excreted in the urine has previously been associated with renal papilla injury from nephrotoxin insult and may play a role in maintaining osmotic balance (Gartland *et al.*, 1989) and thus, this metabolite may serve as a biomarker of indirect renal papilla stress from the renal filtration of muscle products released into the blood stream following resistance-based exercise. Hippurate (anion of hippuric acid and derivative of glycine and benzoic acid) and citrate (anion of citric acid in the TCA cycle) are excreted in the urine in concentrations ranging between 2.79-27.91 mM and 0.39-12.49 mM, respectively (Stedman, 2000), and decreases exhibited in these carboxylic acids, together with the amino acid glycine, may be representative of acute renal tubular injury (Bairaktari *et al.*, 2002). Pechlivanis *et al.* (2010) suggested that exercise-induced lactate acidosis may cause renal tubular acidosis, hence the link to renal stress.

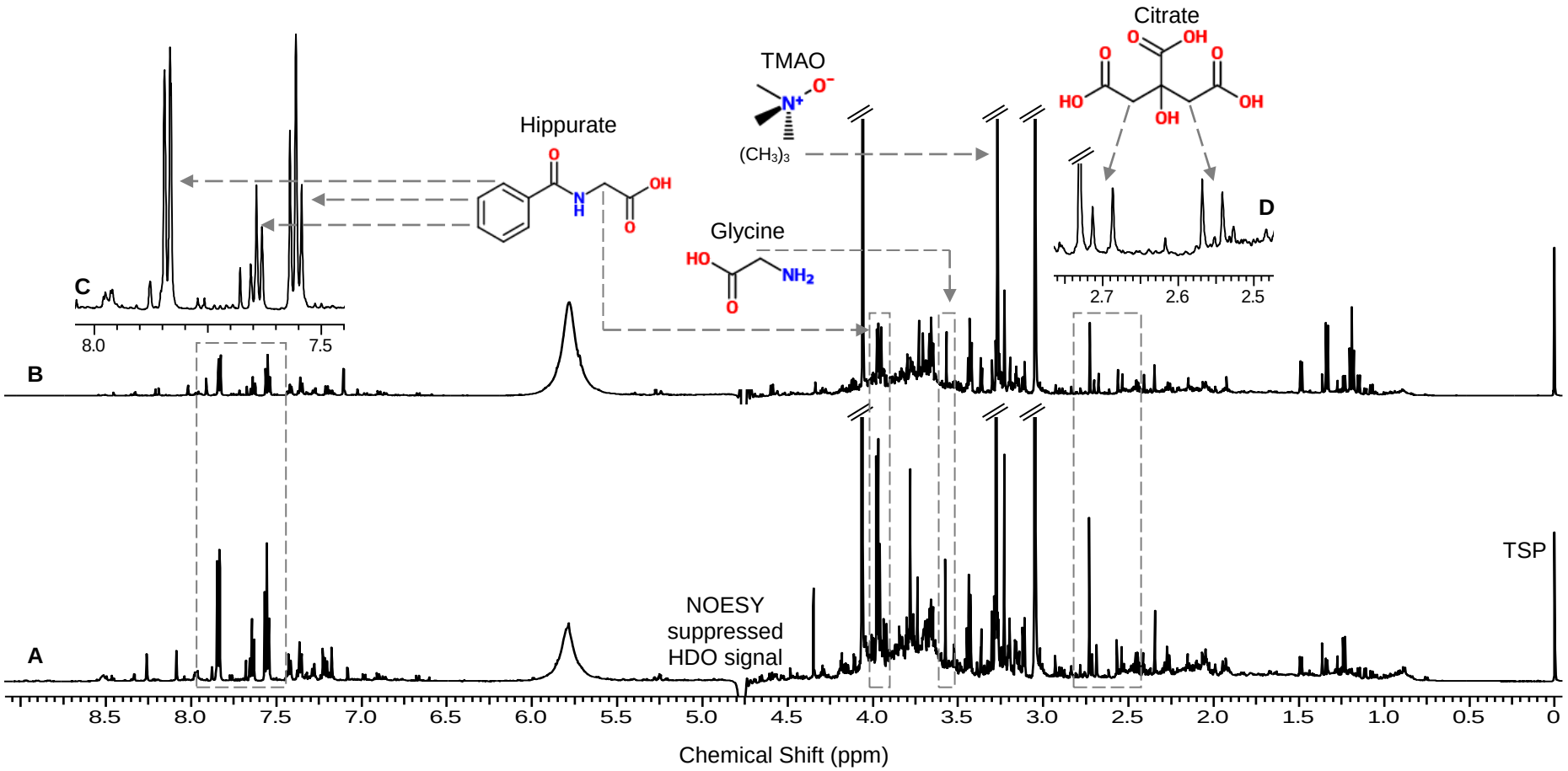


Figure 4.9 600 MHz ^{1}H NMR spectra of urine taken **(A)** pre- and **(B)** post-HIE from one participant showing regions δ 0.00-9.00, with expanded regions of **(C)** δ 2.50-2.75 and **(D)** 7.50-8.00. Signals assigned to metabolites potentially associated with muscular exercise-induced modifications in renal function have been labelled with reference to the molecular structures. TMAO = Trimethylamine *N*-oxide.

4.4 Chapter Conclusion

This chapter demonstrates the value of urinalysis by 600MHz ^{1}H NMR spectroscopy and chemometrics to profile change in urinary metabolites induced by concentric isokinetic exercise of the knee extensors and flexors at 80%MVC and 40%MVC intensities. At both exercise intensities, the results revealed a 2.13-fold increase in urinary trimethylamine *N*-oxide content, with a decrease in urinary hippurate (3.67-fold), citrate (1.67-fold) and glycine (2.13-fold) following concentric muscle loading. This metabolic pattern has been previously associated with myoglobinuria-induced renal papilla and tubular injury from rhabdomyolysis (Bairaktari *et al.*, 2002) and renal tubular acidosis following sprint running (Pechlivanis *et al.*, 2010). However, there was no statistically significant difference in urinary creatinine following HIE and LIE, which suggests that isokinetic exercise did not modify glomerular filtration rates despite the change exhibited in the urinary markers of renal stress, since glomerular function is independent of papilla and tubular stress (Bairaktari *et al.*, 2002).

Such data may provide diagnostic and prognostic information on injured and recovering athletes to determine whether or not they are fit to play, and predict risk of injury based on their current state of health as indicated by low-molecular weight markers. However, the interpretation of the results in this study are speculative and further work is required to fully quantify the urinary concentrations of trimethylamine *N*-oxide, hippurate, citrate and glycine obtained in a resistance-based exercise setting and determine any correlation with known biomolecular markers of muscular stress (e.g. serum myoglobin and CK).

A limitation of urinalysis in sports medicine is that biomarkers have different, and in some cases multiple, biological origins and hence, urinary constituents (pooled) may only serve as indirect biomarkers of muscle metabolism and direct biomarkers of renal function and health. Nevertheless, the composition of urine has shown potential to reflect systemic responses to cycling (Enea *et al.*, 2010; Mukherjee *et al.*, 2014), sprint running (Pechlivanis

et al., 2010), or a combination of cycling and running (Daskalaki *et al.*, 2015), snowboarding training (Wang *et al.*, 2015) and isokinetic concentric exercise (results of this chapter).

The osmolality levels varied extensively between participants meaning that the hydration status varied. The state of hydration affects the glomerular filtration rate and overall health of the kidney (Anastasio *et al.*, 2001) and thus, standardising the water intake prior to urine sampling should be considered in future exercise metabolomic studies, particularly because hydration is important for optimal muscle performance. Paracetamol metabolites were detected in the urine of one individual sampled in the HIE session of this study, which may be attributed to the limited of information retrievable from the PAR-Q forms since it specifically questions the use of prescribed medication as opposed to drugs bought over the counter.

CHAPTER 5: ^{1}H NMR SPECTROSCOPY RESULTS OF BLOOD SERUM SAMPLES AND CHEMOMETRIC MODELLING OF HIE AND LIE

5.1 Background

Blood plasma is the biofluid portion of whole blood which accounts for 55% of the total volume and serves the function of transportation of cells, molecular compounds and electrolytes to and from organs to maintain biological homeostasis (Tortora and Derrickson, 2005). Plasma may be separated from blood cells via centrifugation and extracting the fluid supernatant following the addition of an anticoagulant, such as lithium heparin. The plasma matrix is highly complex due to its constituents of the proteins albumin (transporters), globulins (immune system) and fibrinogen (clotting factor), and hormones, enzymes, low-molecular weight compounds (nutrients and metabolites) and other solutes (gases, salts and ions) (Tortora and Derrickson, 2005). The plasma content of the proteins myoglobin and CK is reflective of muscle membrane leakage during stress or microtrauma and thus, serve as direct biomolecular markers of muscular stress during isokinetic exercise, as measured by enzyme-linked immunoabsorbant assay (Speranza *et al.*, 2007). However, the behaviour of low-molecular weight metabolites in plasma or serum during muscle contractions, beyond targeted lactate measurements, has received limited attention in isokinetic exercise studies.

The blood plasma and serum matrix have been commonly used over, or in conjunction with, other biofluid matrices in ^{1}H NMR-based metabolomic studies on cycling and stress tests on clinical populations. Briefly, blood serum is obtained in the same process as plasma except the sample is permitted to coagulate, in the absence of an anticoagulant, prior to centrifugation to remove the clotting factor (e.g. fibrinogen). Although plasma and serum have been utilised in ^{1}H NMR-based metabolomic studies on exercise, no clear rationale was reported to support the choice of specimen, possibly because both matrices contain low-molecular weight blood metabolites of interest.

To the author's knowledge, Coolen *et al.* (2008) were the first group to apply ^{1}H NMR spectroscopic analysis of plasma and urine in conjunction with conventional biochemical assays to study the effects of vitamin C and E supplementation during treadmill walking on patients with intermittent claudication. The conventional assays utilised in the study targeted biomarkers of oxidative stress (isoprostanes, paraoxonase, dityrosine, 8-hydroxy-2'-deoxyguanosine and ferric-reducing ability of plasma), inflammatory response (interleukin-6, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, soluble E-selectin and C-reactive protein), endothelial damage (endothelin-1, von Willerbrand factor, nitrite/nitrate and thrombomodulin), coagulation (fibrinogen, D-dimers, antithrombin, plasmin-antiplasmin, thrombin-antithrombin, and factor-7), organ damage (micro-albumin) and supplement compliance (Vitamins C and E). The study reported that vitamin supplementation enhanced metabolic recovery from exercise for these patients based on the ^{1}H NMR spectroscopy results, which elucidated multiple biomolecules associated with glycotic pathways (lactate, alanine and glucose), ketogenesis (acetoacetate) and the tricarboxylic acid (TCA) cycle (citrate and 2-oxoglutarate), whereas limited change was reported in the targeted biomarkers. Also in 2008, Barba *et al.* (2008) performed a ^{1}H NMR spectroscopy study on patients with suspected coronary artery disease and were able to discriminate between patients with and without exercise-induced ischemia based on differences in serum lactate, glucose, CH_3 and CH_2 moieties of lipids, and BCAA. By 2012, two further clinical ^{1}H NMR-based metabolomic studies on cycling exercise were conducted on patients with chronic obstructive pulmonary disease (COPD) (Rodriguez *et al.*, 2012) and type I diabetes (Brugnara *et al.*, 2012). Rodriguez *et al.* (2012) reported that the variable discriminators between COPD patients and healthy controls, following 8 weeks of training, were plasma BCAA at rest, and alanine, succinate and creatine after a single session of cycling. Brugnara *et al.* (2012) reported that the variable discriminators between diabetics and healthy controls following 30 min of cycling included serum intermediates of the tricarboxylic acid cycle (citrate, malate, fumarate, succinate, α-ketoglutarate and α-ketoisocaproate) and glycotic pathways (alanine, lactate and glycerol). The outcome of these exercise studies provided

metabolomic data informative of functional restoration of clinical populations undergoing physiotherapy and how these exercise responses compare to healthy controls.

In addition, non-clinical ^{1}H NMR-based metabolomic studies have been published on sports drink-enhanced recovery of athletes following rowing exercise (Miccheli *et al.*, 2009), acute metabolic responses to exhaustive cycling (Kirwan *et al.*, 2009), chronic metabolic adaptations to sprint running training (Pechlivanis *et al.*, 2013) and metabotyping of populations based on physical activity participation (Kujala *et al.*, 2013). These studies demonstrate the versatility of ^{1}H NMR spectroscopy as means to study metabolic responses and adaptations to endurance-based exercise and sprint training, and discriminate between cohorts accustomed to an active lifestyle. However, the effect of resistance-based exercise on plasma or serum metabolites has yet to be investigated by a comprehensive metabolomic profiling platform.

Due its biomolecular complexity, plasma and serum are interesting yet challenging matrices for ^{1}H NMR spectroscopy profiling since the macromolecular compounds present give rise to broad resonances caused by the relatively short T_2 relaxation times of macromolecular protons (Daykin *et al.*, 2002). To overcome such broad resonances, previous metabolomic studies on exercise applied a deproteinization method to plasma by ultrafiltration using 10-kD centrifugal filters during sample preparation, followed by routine pulse-acquire experiments (Miccheli *et al.*, 2009; Rodriguez *et al.*, 2012). However, blood plasma proteins below the 10-kD size may have been retained in the sample. The limitation of plasma deproteinization by ultrafiltration is the subsequent poor ^{1}H NMR signal-to-noise ratio compared to intact plasma analysis, possibly related to the lack of separation between low-molecular weight metabolites and macromolecules (Daykin *et al.*, 2002). Daykin *et al.* (2002) reported that blood plasma deproteinization by acetonitrile precipitation at a physiological pH (7.4) retained higher concentrations of metabolites in comparison to ultrafiltration, which is a consideration for prospective studies whereby deproteinization is necessary. As an alternative to lengthy extraction methodologies, the broad macromolecular

resonances can be attenuated by employing CPMG spin-echo pulse sequences with appropriate T_2 relaxation delays on intact blood plasma or serum (Nicholson *et al.*, 1995). The majority of ^{1}H NMR-based metabolomics on exercise favoured CPMG-edited spectra over deproteinization techniques (Barba *et al.*, 2008; Coolen *et al.*, 2008; Kirwan *et al.*, 2009; Brugnara *et al.*, 2012; Pechlivanis *et al.*, 2013; Kujala *et al.*, 2013).

To the author's knowledge, there are currently no published data on the effect of concentric muscle loading during isokinetic exercise on blood serum metabolites as measured by ^{1}H NMR spectroscopy. Such data may elucidate direct markers of the metabolic stress associated with concentric muscle contractions which may have implications for prescribing resistance-based exercise and assess exercise tolerance in practice.

5.2 Chapter Aim

To investigate the effect of HIE and LIE on blood serum metabolites by performing ^{1}H NMR spectroscopy experiments on serum samples collected pre- and post-exercise. In an attempt to attenuate the broad resonances of blood proteins, metabolomic data was sourced from serum (clotting factor proteins removed) via CPMG spin-echo experiments.

Objectives:

I. Present illustrative ^{1}H NMR spectra of blood serum, taken from a participant, acquired from a 1D CPMG spin-echo experiment and a routine experiment using the 1D NOESY pulse sequence, to show the results of macromolecular resonance attenuation in the datasets.

II. Perform PCA on the ^{1}H NMR spectral data to investigate the effect of HIE and LIE on blood serum metabolites without a predetermined classifier and detect and manage outlying spectra.

III. Perform PLS-DA on the ^{1}H NMR spectral data to optimise separation of scores according to class and elucidate metabolites potentially associated with muscular stress imposed by HIE and LIE.

5.3 Results and Discussion

5.3.1 ^{1}H CPMG Spin-Echo NMR Spectroscopy of Blood Serum Samples

Throughout this chapter, resonance assignment was carried out according to the literature (Nicholson *et al.*, 1995; Daykin *et al.*, 2001; Psychogios *et al.*, 2011; Pechlivanis *et al.*, 2013) and HMDB (www.hmdb.ca), based on chemical shift values and signal multiplicity according to molecular structures.

As illustrated in Figure 5.1D, the spectra acquired from a routine experiment using the NOESY pulse sequence is highly complex and contains broad resonances of blood proteins (e.g. albumin), lipids and lipoproteins together with sharp signals of low-molecular weight metabolites superimposed on top. The ^{1}H CPMG-edited NMR spectra successfully attenuated the broad resonances of the macromolecules present in blood serum as illustrated in Figure 5.1. However, residual broad signals of low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) were visible in the spectra, which was in agreement with the literature (Nicholson *et al.*, 1995; Barba *et al.*, 2008). Since the spin-echo pulse sequence attenuated macromolecular signals, it is possible that the low-molecular weight, fatty acid components of LDL and VLDL were still detectable, hence the residual broad resonances present in the CPMG-spectra.

As shown in Figure 5.1A-C, there was a visible increase in the lactate signals (δ 1.33 and 4.13) post-isokinetic exercise, indicative of anaerobic energy metabolism, which reduced towards baseline (state of rest) after one hour of exercise cessation, reflective of metabolic recovery. However, the overlap of the CH_3 signal of lactate with the fatty acid components of lipoprotein presents difficulty in the semi-quantification of serum lactate concentration in the samples.

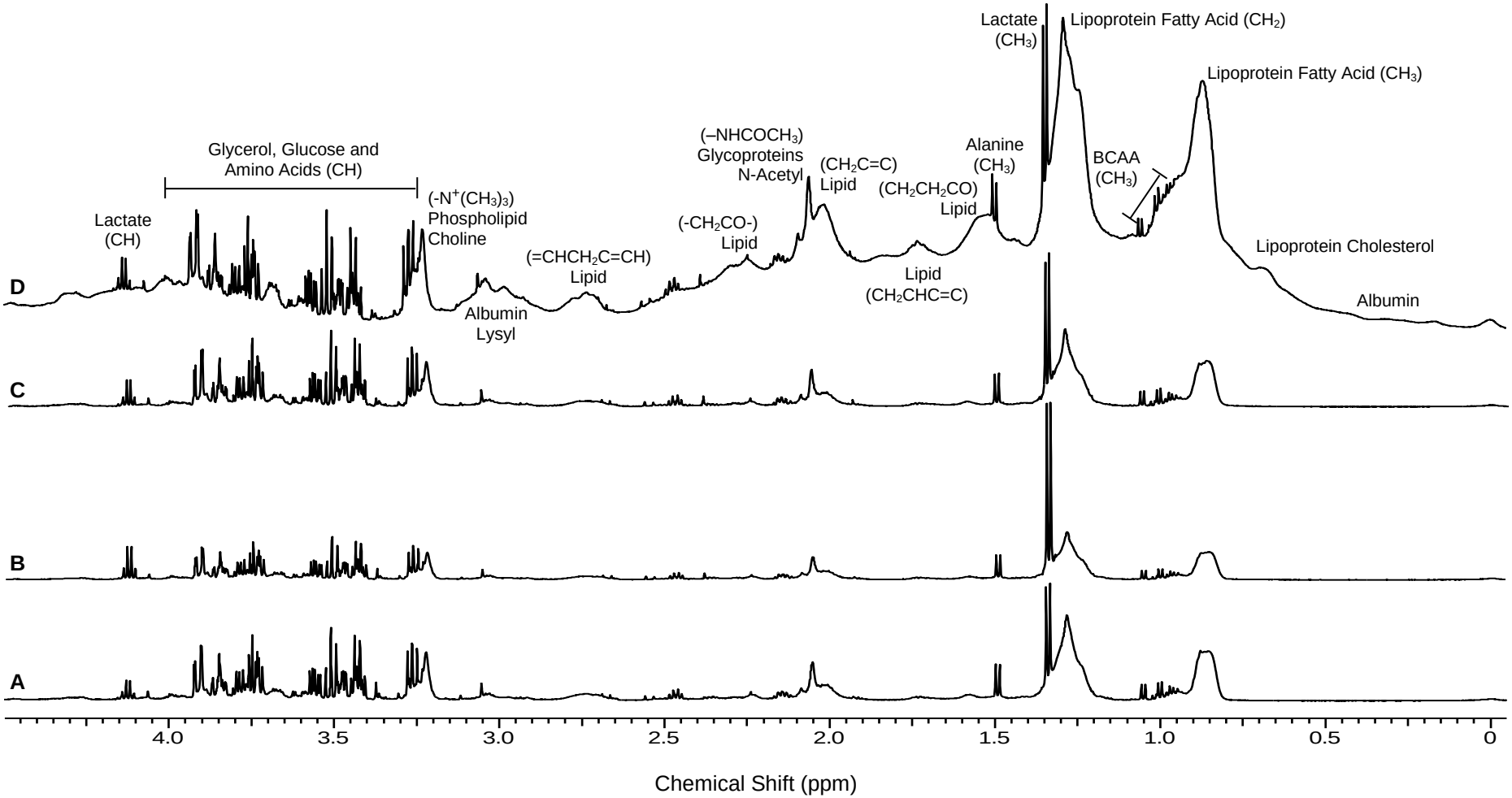


Figure 5.1 Aliphatic (δ 0.0-4.5) 600 MHz ^{1}H CPMG NMR spectra (32 scans) of blood serum taken **(A)** pre-, **(B)** post- and **(C)** 1 hr post-HIE from a participant. **(D)** Spectrum of sample (C) acquired from a routine experiment using the 1D NOESY pulse sequence, showing the influence of macromolecular signals.

5.3.2 The Effect of HIE and LIE on Blood Serum Metabolites

In the present chapter, the 84 blood serum samples are each referred to by a unique identification code: the letters ‘x’, ‘y’ or ‘z’ denotes the point of collection for either pre-, immediately post- or 1 hr post-exercise, respectively; ‘H’ or ‘L’ denotes either the high or low intensity exercise session, respectively, from which the sample was collected; the numbers ‘01-14’, denotes the participant number. PCA was performed on the 600 MHz ^{1}H NMR spectra to investigate the effect of isokinetic exercise on blood serum metabolites without the influence of a predetermined classifier (unsupervised) and to detect potential outlying spectra, since outliers can mathematically skew data models and usually fail to provide information coherent with representative scores.

The PCA models of the HIE and LIE datasets are presented in Figures 5.2 and 5.3, respectively. No statistical outlying spectra were detected in either of the HIE and LIE scores plots since all scores in both models fell within Hotelling’s 95% confidence limits (ellipses). No uniform separation was exhibited according to class in both models since inter-individual variation was greater than the effect of HIE and LIE, due to individual differences at baseline and varying responses to the isokinetic exercise interventions and recovery employed in this study. The loadings plots for the HIE and LIE datasets illustrate that the strongest variable weightings on the scores across PC1 and PC2 included the integrals of the acetyl functional group (CH_3 bonded to a carbonyl group) of glycoprotein (δ 2.06), the CH_3 lactate signal (δ 1.33), fatty acid CH_3 signals of LDL (δ 1.23 or 1.26 and 0.84) and VLDL (δ 1.28 and 0.87). Signals of glycerol, glucose and CH protons of amino acids (δ 3.00-4.00) were also elucidated by the loadings of HIE dataset. However, there is no confirmation that these metabolites are directly associated with concentric isokinetic exercise at this stage, since this finding is based on an unsupervised model with no class separation exhibited and hence, supervised chemometric analysis was employed for such verification.

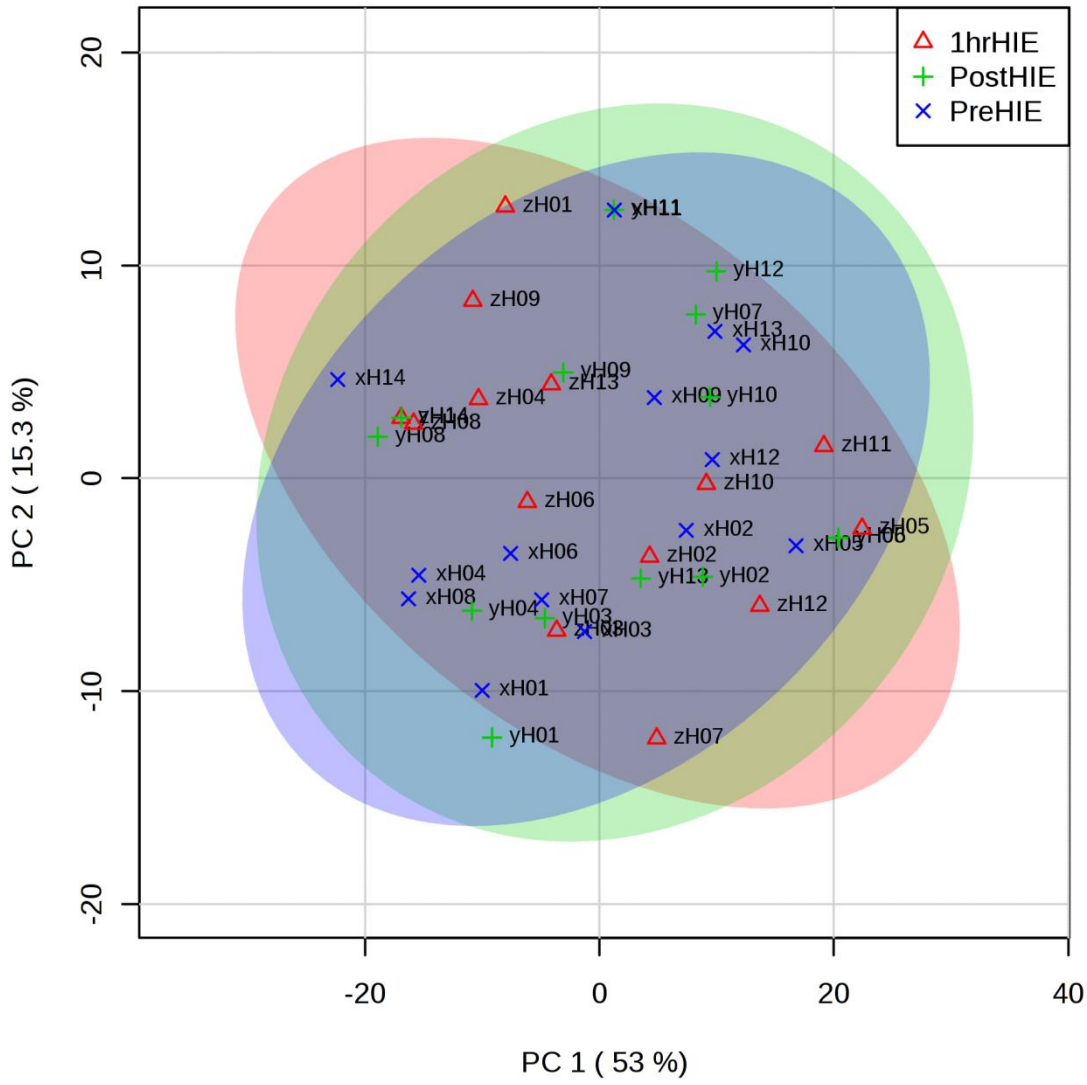


Figure 5.2A HIE Unsupervised Model: PCA scores plot of 600 MHz ^{1}H NMR spectra of blood serum, normalised to sum of intensities, taken from 14 healthy men (x) pre-, (+) immediately post- and (Δ) 1 hr post-HIE. PC1 and PC2 explain 53.1% and 15.3% of variance, respectively. Throughout this chapter, the blue, green and red ellipses in all data models denote the 95% confidence regions for the pre-, post- and 1 hr post-exercise scores, respectively. No outliers were elucidated since all scores fell within Hotelling's 95% confidence limits. Inter-individual variation was greater than the effect of exercise.

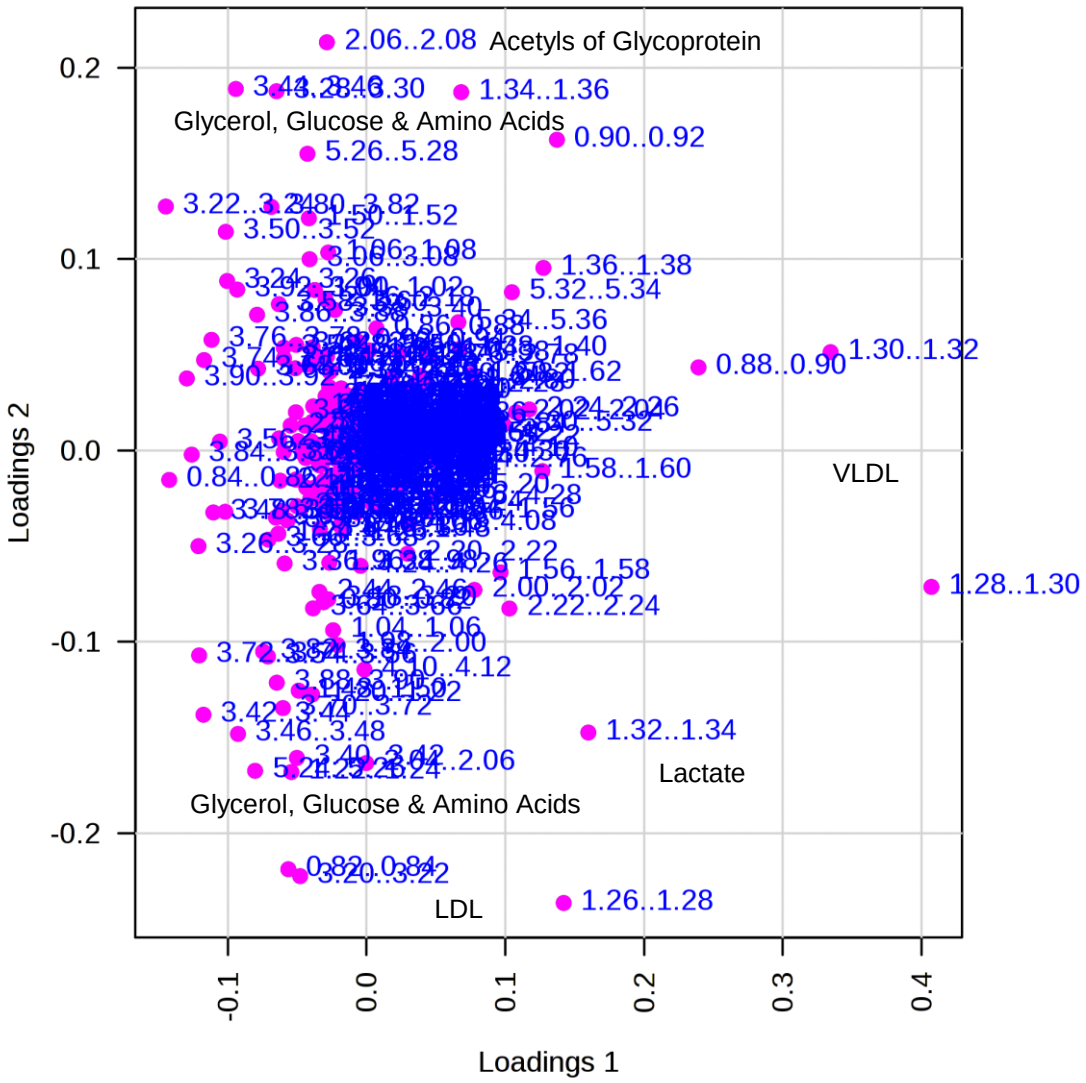


Figure 5.2B HIE Unsupervised Model: PCA loadings plot showing the weighting of variables (spectral integrals) on the HIE scores. The variables were assigned to the acetyl group of glycoprotein (δ 2.06), the CH_3 signal of lactate (δ 1.33), fatty acids of LDL (δ 1.26 and 0.84) and VLDL (δ 1.28 and 0.87), and glycerol, glucose and CH protons of amino acids (δ 3.00-4.00).

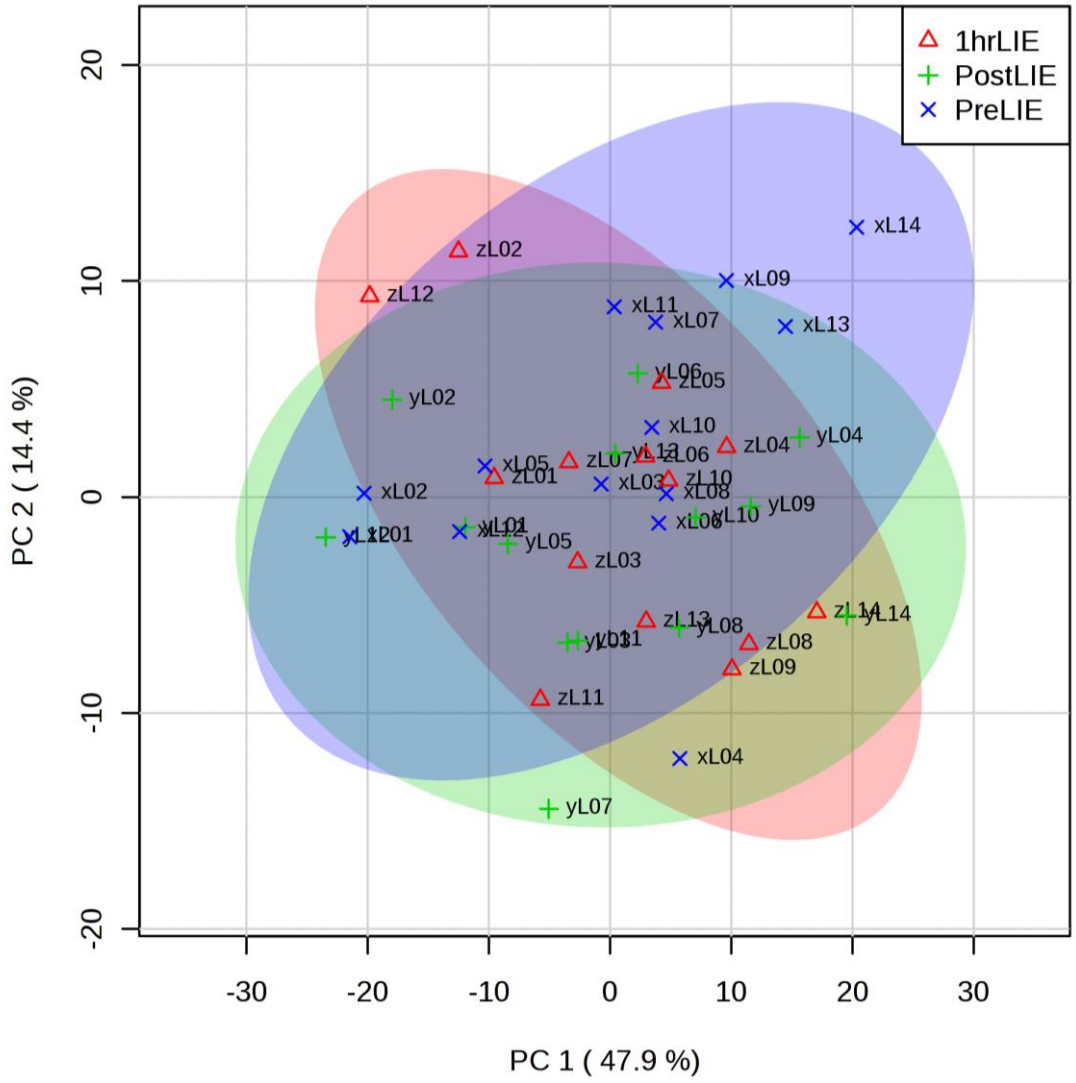


Figure 5.3A LIE Unsupervised Model: PCA scores plot of 600 MHz ^{1}H NMR spectra of blood serum, normalised to sum of intensities, taken from 14 healthy men (x) pre-, (+) immediately post- and (Δ) 1 hr post-LIE. PC1 and PC2 explain 47.9% and 14.4% of variance, respectively. No outliers were elucidated since all scores fell within Hotelling's 95% confidence limits. Inter-individual variation was greater than the effect of exercise.

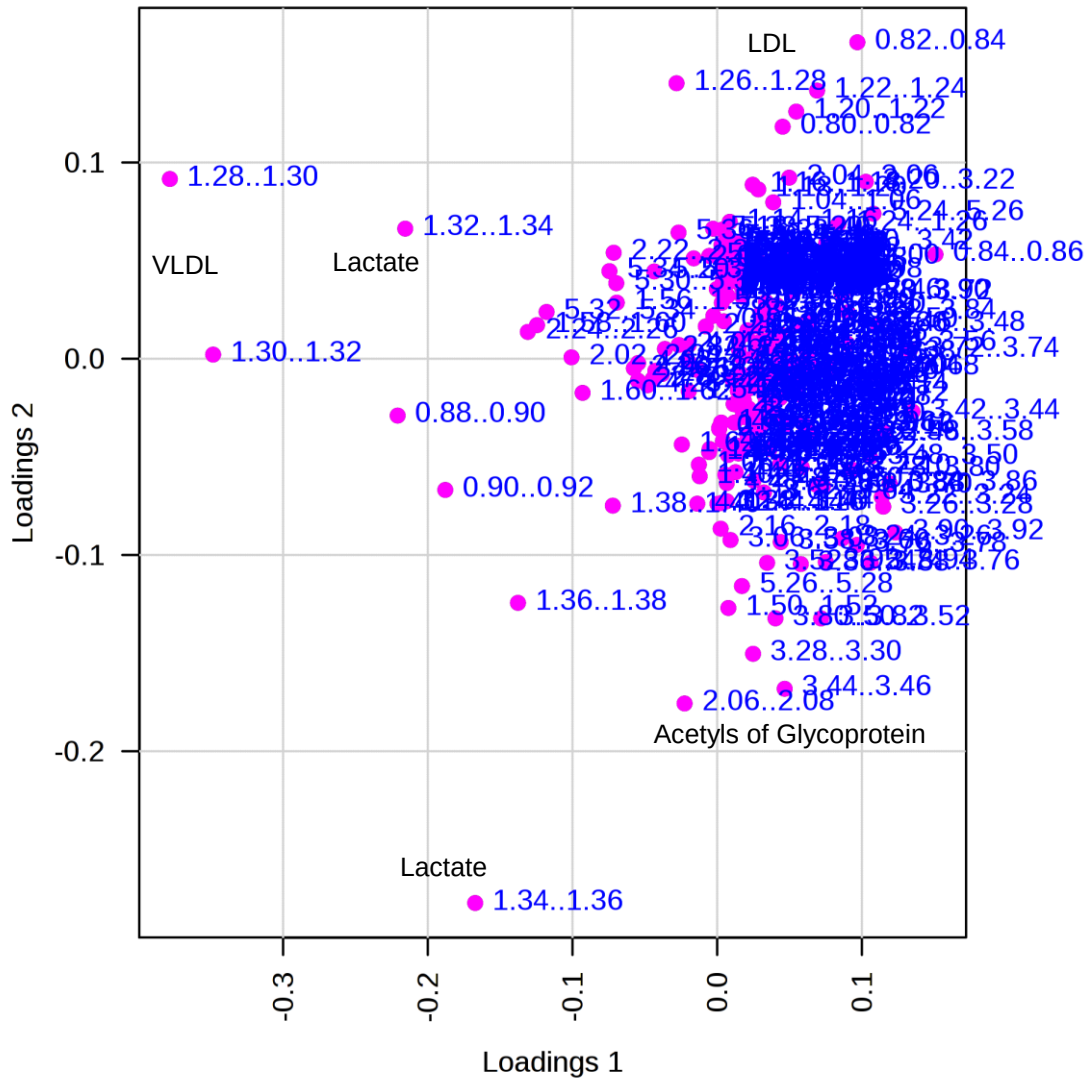


Figure 5.3B LIE Unsupervised Model: PCA loadings plot showing the weighting of variables (spectral integrals) on the LIE scores. The variables were assigned to the acetyl group of glycoprotein (δ 2.06), the CH_3 signal of lactate (δ 1.33), and fatty acids of LDL (δ 1.23 and 0.84) and VLDL (δ 1.28 and 0.87).

Since PCA did not reveal any clear uniform responses towards HIE and LIE, and recovery, PLS-DA was performed on the serum spectral datasets to discriminate between classes with exercise as the classifier (supervised) and to elucidate spectral integrals associated with the classifier to identify metabolites potentially allied to metabolic responses to HIE and LIE.

Figure 5.4A presents the PLS-DA scores plot of the HIE dataset, whereby component 1 and 2 explain 30.4% and 30.5% of variance, respectively. No class distinction was achieved due to a high degree of overlap between classes since inter-individual variation exceeded the effect of HIE. Nevertheless, a subtle separation was exhibited between pre-, immediately post- and 1 hr post-HIE scores for each individual, which represents individual responses to HIE and recovery as opposed to a uniform group response. In an attempt to improve class separation, a third component vector (explaining 7.1% of variance) was included in the PLS-DA model and as a result, separation improved slightly between classes; however, a high degree of class overlap remained (Figure 5.4B). The PLS-DA loadings associated with the classifier on components 1 and 2 were the integrals of lactate (δ 1.33 and 4.13) and fatty acids of VLDL (δ 1.28 and 0.87) (Figure 5.4C), and the loadings on component 3 were integrals of the acetyl functional group of glycoprotein (δ 2.06) (Figure 5.4D).

Figure 5.5A presents the PLS-DA scores plot of the LIE dataset, whereby component 1 and 2 explain 15% and 38.1% of variance across, respectively. No class distinction was achieved due to a high degree of overlap between classes since inter-individual variation exceeded the effect of LIE and recovery however, separation between classes improved slightly after the inclusion of a third component vector explaining 12.8% of variance (Figure 5.5B). Consistent with the HIE data model, the loadings associated with the classifier on components 1 and 2 were the integrals of lactate and fatty acids of VLDL (Figure 5.5C). The loadings on component 3 were integrals of fatty acids of LDL (Figure 5.5D).

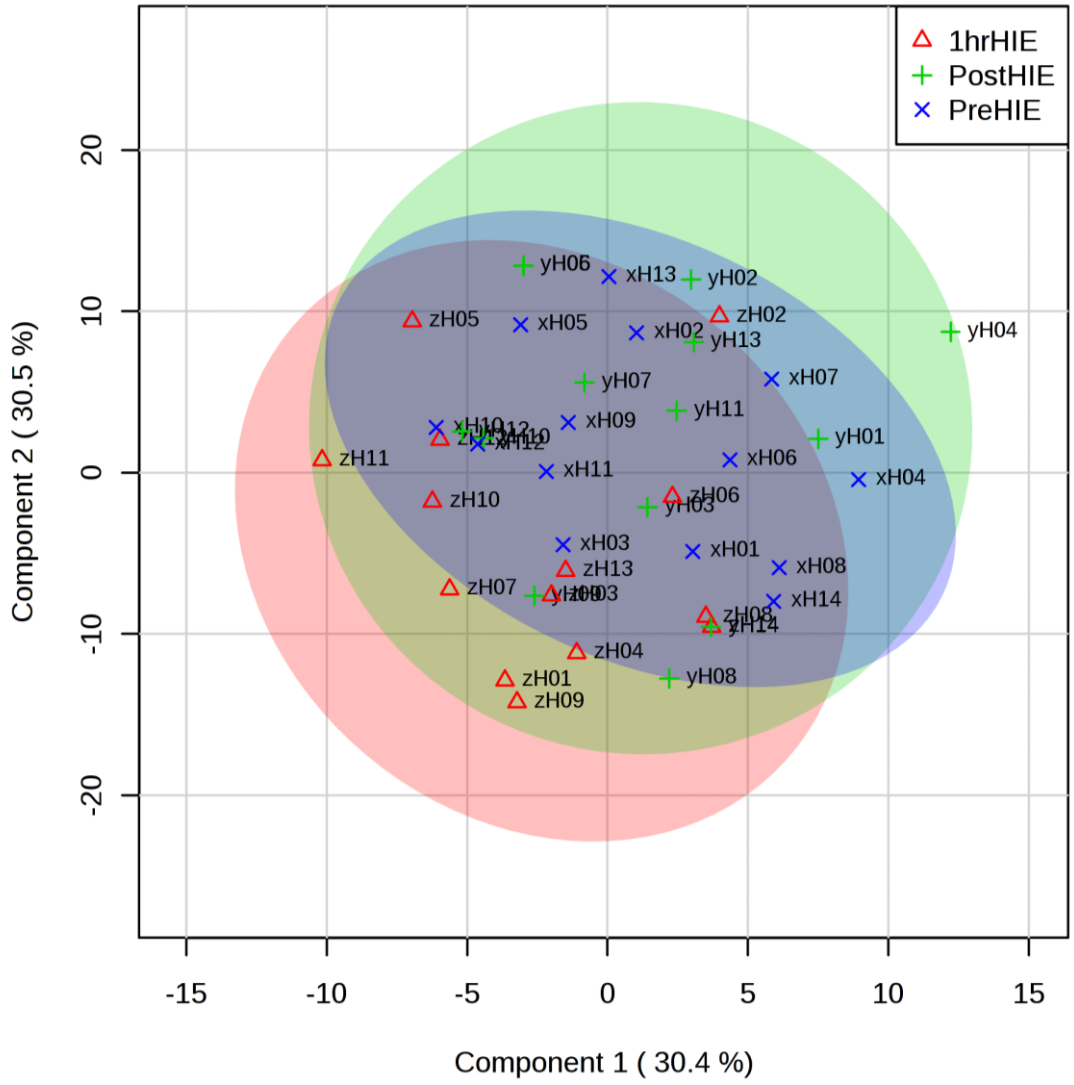


Figure 5.4A HIE Supervised Model: PLS-DA scores plot of 600 MHz ^{1}H NMR spectra of blood serum, normalised to sum of intensities, taken from 14 healthy men (x) pre-, (+) immediately post- and (Δ) 1 hr post-HIE. Component 1 and 2 explain 30.4% and 30.5% of variance, respectively. No class distinction was established due to the overlapping scores between the classes caused by inter-individual variation.

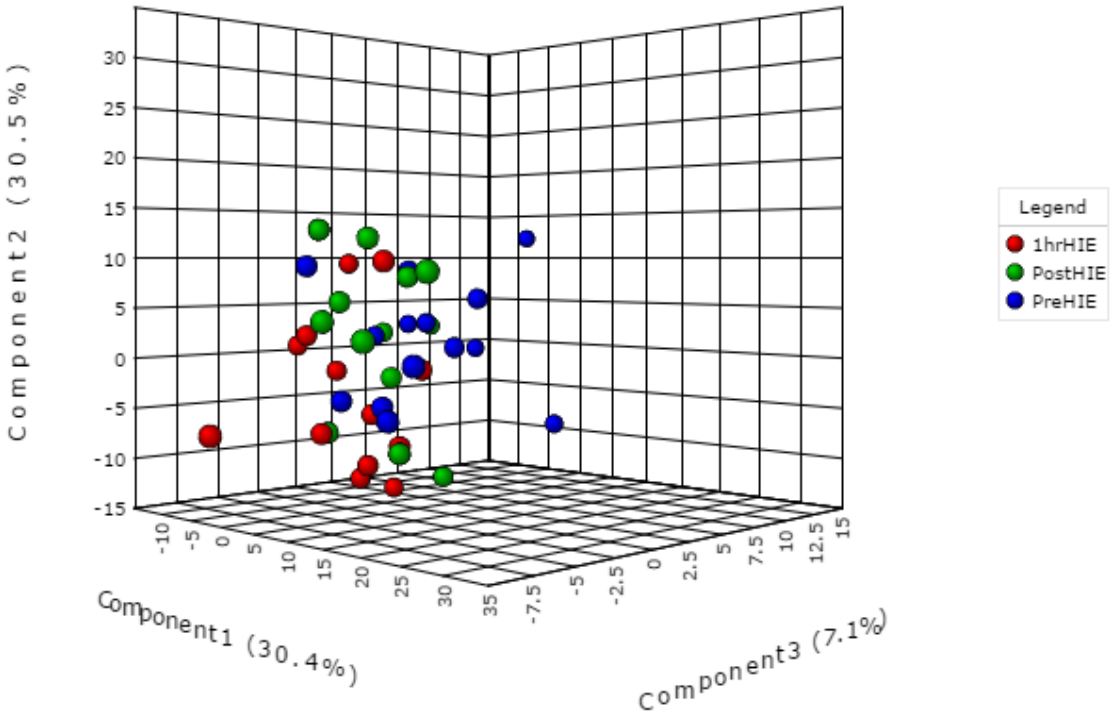


Figure 5.4B HIE Supervised Model: PLS-DA scores plot of the HIE dataset with the inclusion of a third component vector. Component 1, 2 and 3 explain 30.4%, 30.5% and 7.1% of variance, respectively. The blue, green and red circles denote scores representative of blood serum sampled pre-, immediately post- and 1 hr-post HIE, respectively. A subtle separation is observable between the classes (shift from right to left after exercise); however, a complete class distinction was not achieved due to the overlap caused by inter-individual variation.

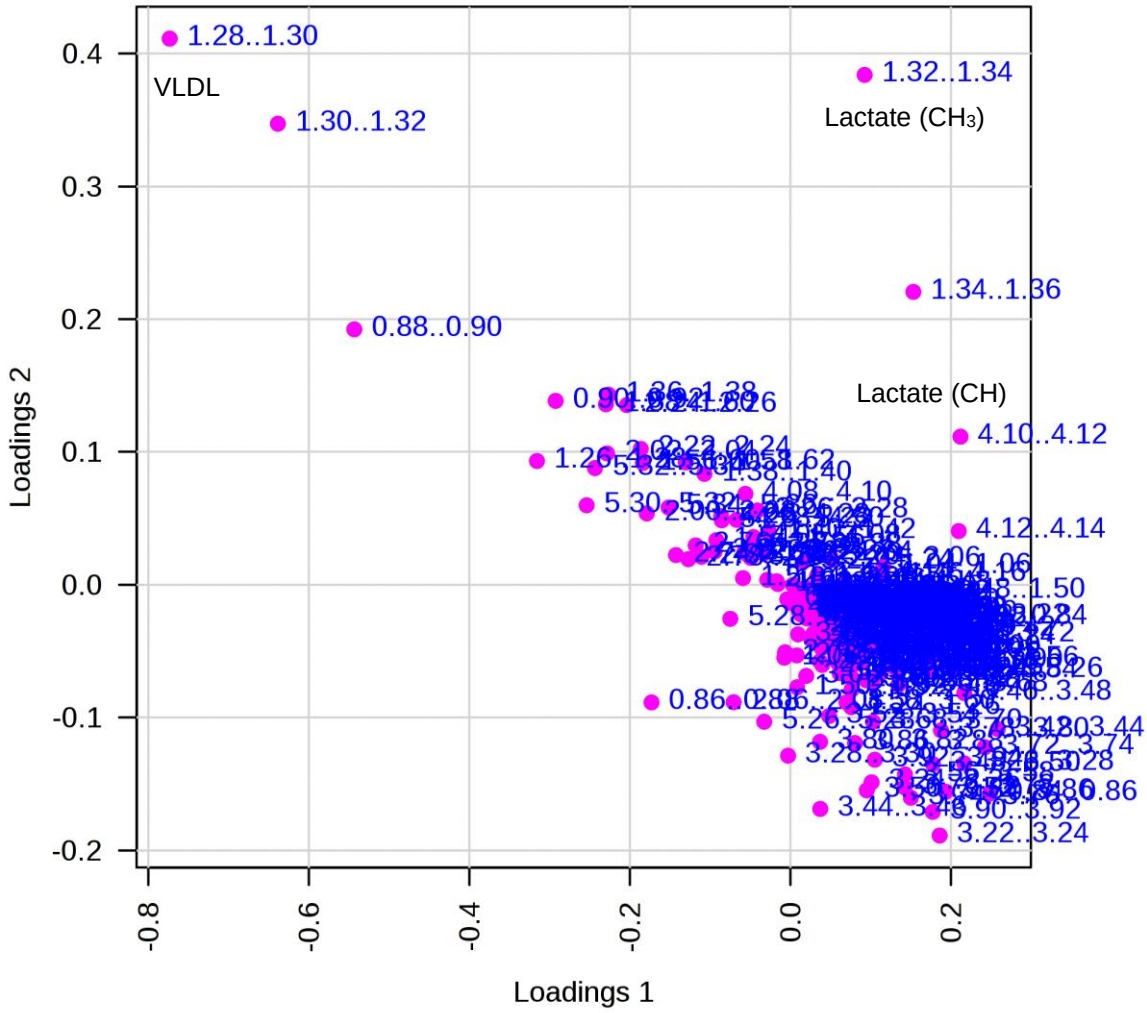


Figure 5.4C HIE Supervised Model: PLS-DA loadings plot for components 1 and 2 showing the weighting of variables (spectral integrals) on the HIE scores. The variables associated with the classifier were assigned to lactate (δ 1.33 and 4.13) and fatty acids of VLDL (δ 1.28 and 0.87).

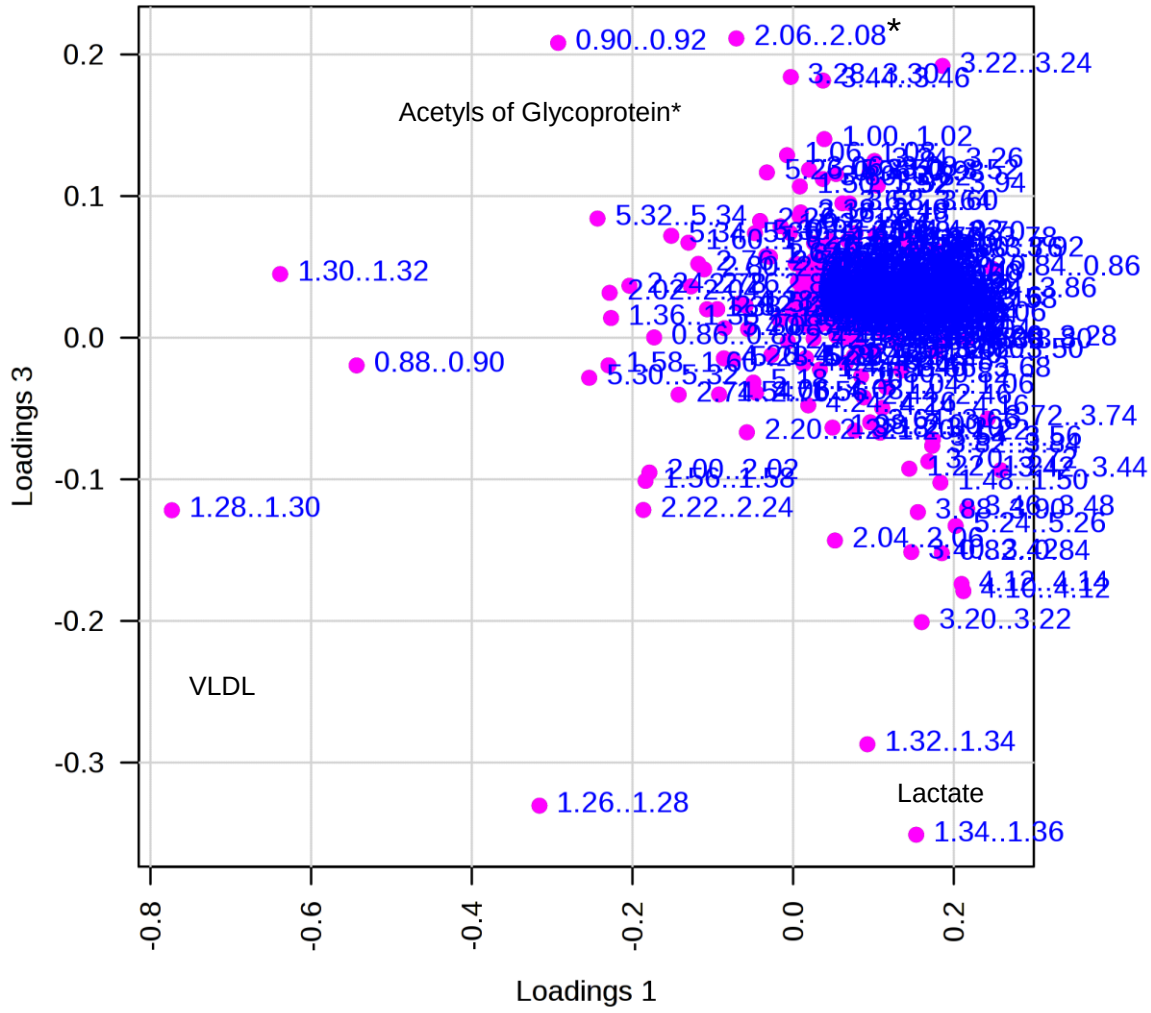


Figure 5.4D HIE Supervised Model: PLS-DA loadings plot for components 1 and 3 of the HIE dataset. Integrals were assigned to acetyls of glycoprotein (δ 2.06), lactate (δ 1.33) and VLDL (δ 1.28 and 0.87).

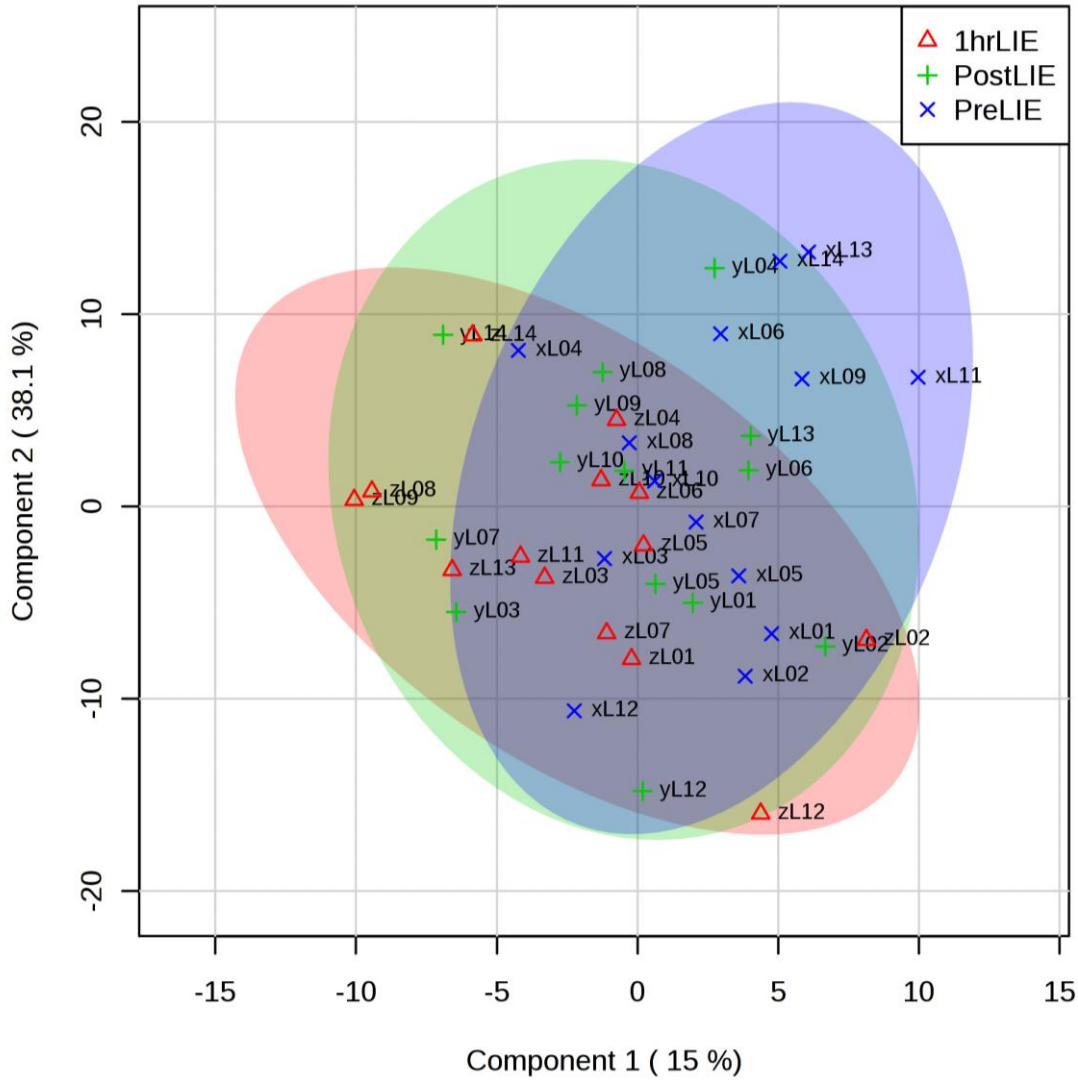


Figure 5.5A LIE Supervised Model: PLS-DA scores plot of 600 MHz ^{1}H NMR spectra of blood serum, normalised to sum of intensities, taken from 14 healthy men (x) pre-, (+) immediately post- and (Δ) 1 hr post-LIE. Component 1 and 2 explain 15% and 38.1% of variance, respectively. No class distinction was established due to the overlapping scores between the classes caused by inter-individual variation.

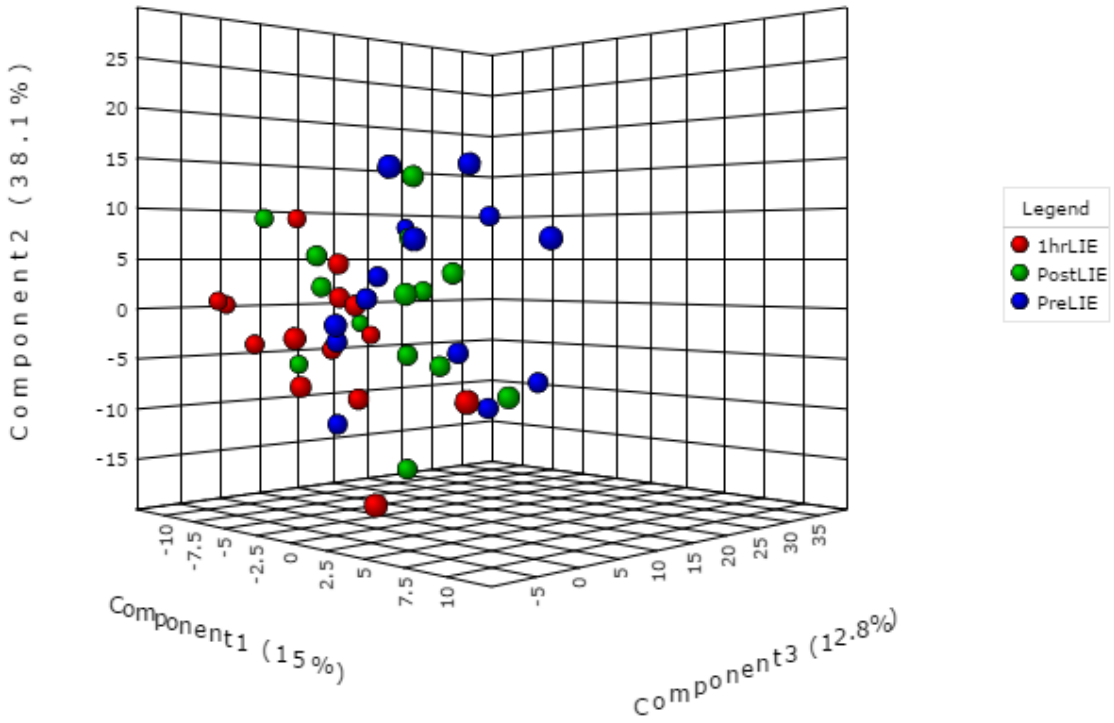


Figure 5.5B LIE Supervised Model: PLS-DA scores plot of the LIE dataset with the inclusion of a third component vector. Component 1, 2 and 3 explain 15%, 38.1% and 12.8% of variance, respectively. The blue, green and red circles denote scores representative of blood serum sampled pre-, immediately post- and 1 hr-post LIE. A subtle separation is observable between the classes (shift from right to left after exercise); however, a complete class distinction was not achieved due to the overlap caused by inter-individual variation.

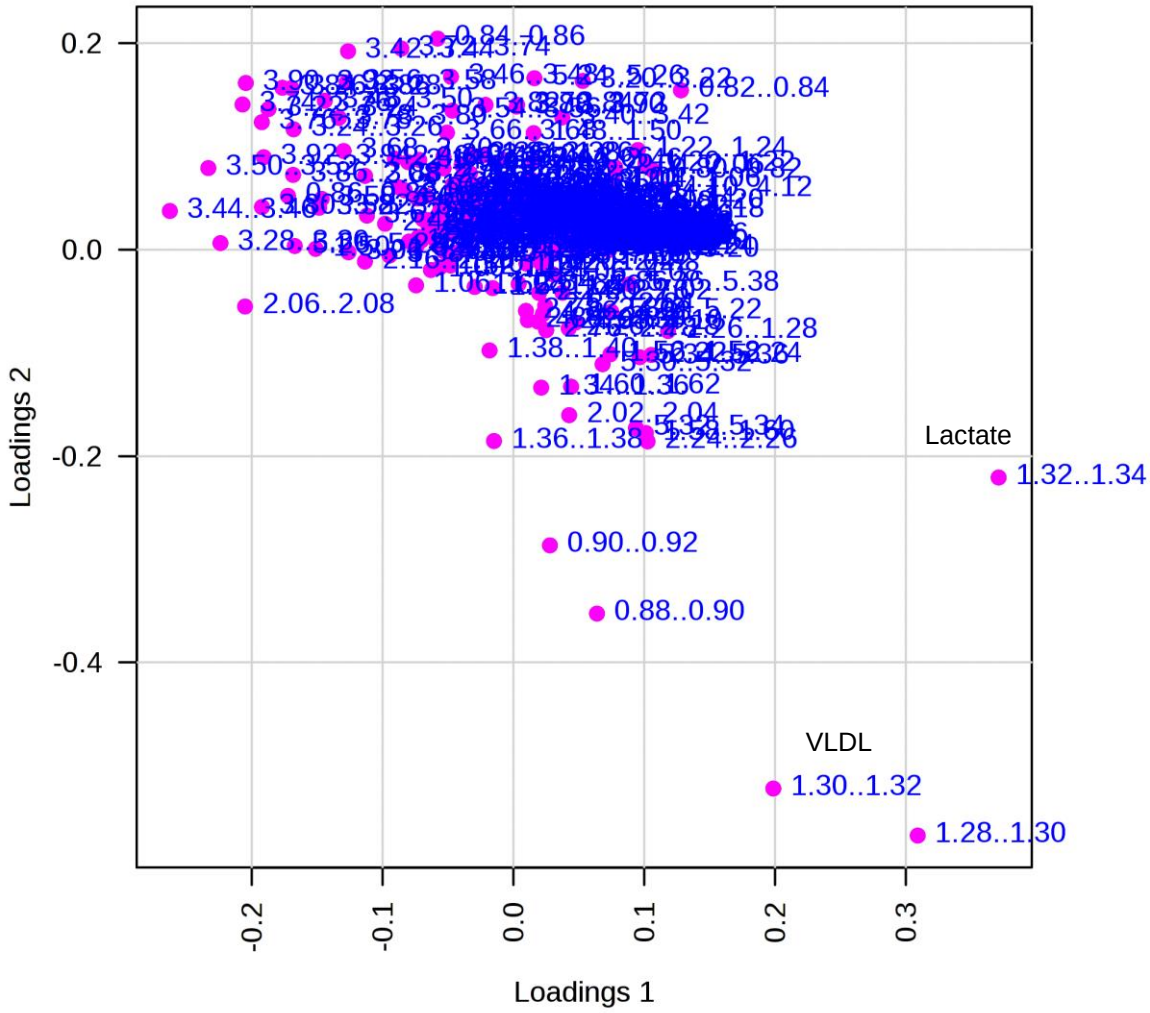


Figure 5.5C LIE Supervised Model: PLS-DA loadings plot for components 1 and 2 showing the weighting of variables (spectral integrals) on the LIE scores. The variables associated with the classifier were assigned to lactate (δ 1.33) and VLDL fatty acids (δ 1.28 and 0.87).

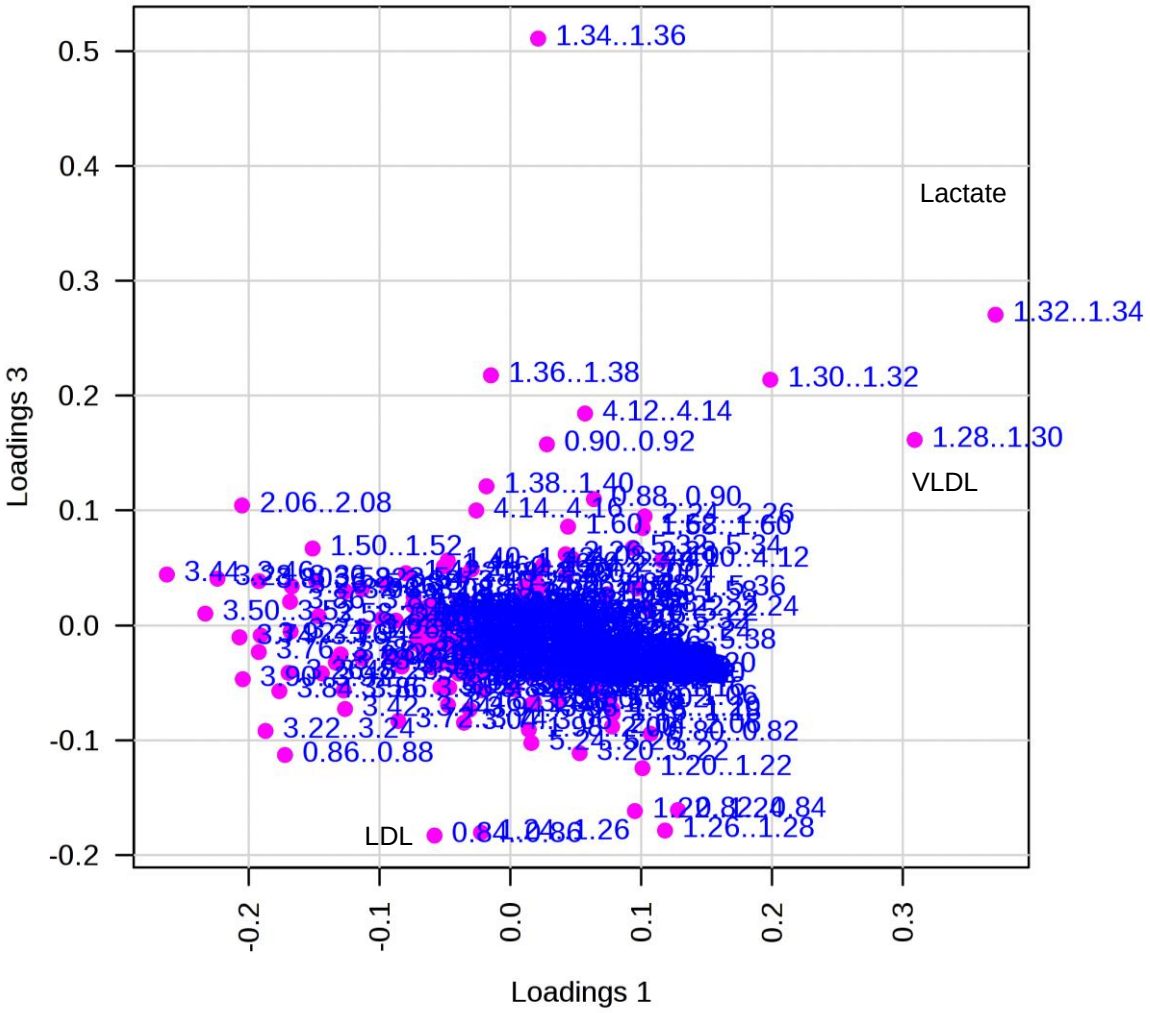


Figure 5.5D LIE Supervised Model: PLS-DA loadings plot for components 1 and 3 of the LIE dataset. Integrals associated with the classifier were assigned to fatty acids of LDL (δ 1.23 and 0.84) and VLDL (δ 1.28 and 0.87), and lactate.

Serum lactate was a strong variable weighting on the discrimination between classes relative to other serum molecular components in both the HIE and LIE data models. ^{1}H NMR signals of lactate increased immediately-post exercise, more so in some individuals than others, and then reduced towards baseline one hour post-exercise as muscle metabolism returned towards rest, as illustrated in Figure 5.1. Although speculative, the inter-individual differences in serum lactate accumulation and removal may be explained by inter-individual differences in plasma content of monocarboxylate transporters (MCTs) to cotransport lactate and protons (H^{+}) across the sarcolemma to maintain muscle pH (Thomas *et al.*, 2012). During the recovery phase of isokinetic exercise, excess lactate molecules may have been converted into glucose via the gluconeogenesis pathways, as evidenced by the reduction exhibited in blood serum lactate signals and an increase in the glucose anomeric signal (δ 5.26) one hour after exercise cessation (Figure 5.6).

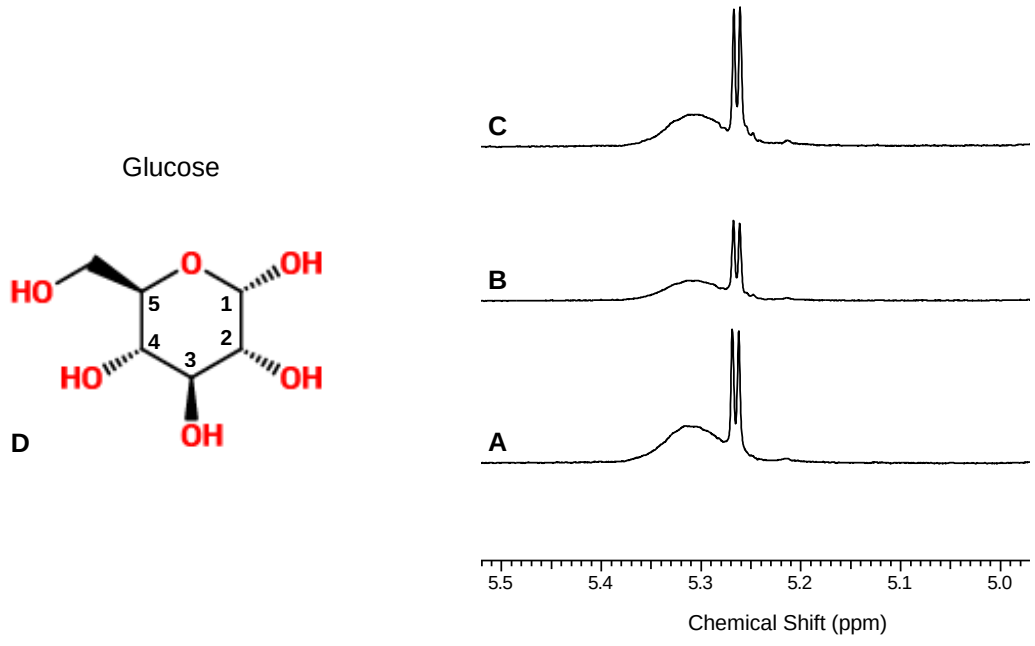


Figure 5.6 The region of δ 5.00-5.50 of 600MHz ^{1}H CPMG NMR spectra (32 scans) of blood serum taken from a participant, showing the glucose anomeric signal, **(A)** pre- **(B)** post- and **(C)** 1 hr post-HIE. **(D)** Glucose molecule giving rise to the doublet signals at (δ 5.26).

Blood lactate concentration is routinely measured in exercise science by lactate assays to serve as a biomarker of anaerobic glycolysis representative of exercise intensity and tolerance (Kass and Carpenter, 2009). Previous studies on isokinetic exercise (similar to the interventions employed in this study) have reported a 2.13-fold increase in lactate concentration in capillary whole-blood (Marzorati *et al.*, 2000), and a 9.25-fold and 13.64-fold increase in blood serum sampled from endurance and power athletes, respectively (Rainoldi *et al.*, 2008). However, the accuracy of semi-quantification of serum lactate in the present study was limited by the CH_3 signal overlap with CH_2 protons of VLDL/LDL fatty acids, despite the use of CPMG-edited spectra to attenuate the broad signals of serum macromolecules and hence, no numerical values of lactate levels were reported here. In fact, Daykin *et al.* (2002) suggested that full-quantification of ^{1}H NMR signals present in CPMG-edited spectra may be impractical due to the metabolite line-broadening effect from the shortened T_2 relaxation times caused by the binding interactions with macromolecules in blood plasma/serum.

The macromolecular signals of fatty acids of LDL and VLDL were also strong variable weightings on the classifier. Exercise-induced modifications in muscle and blood lipoprotein content related to oxidative stress caused by perturbations in the balance of reactive oxygen species (ROS) have been reviewed previously (Aldred, 2007; Nikolaidis *et al.*, 2008). Briefly, ROS are reactive molecules (e.g. superoxide, singlet oxygen, hydrogen and peroxide) that are naturally present in biological tissues and fluids as a result of cellular oxidation processes in normal physiology. Disturbances in the balance of ROS, such as muscular stress or exercise, may cause lipid peroxidation and protein oxidation, which may therefore modify the LDL and VLDL content in blood serum. In fact, Bailey *et al.* (2004) reported marked increases in venoarterial concentration differences in the ROS lipid peroxides following knee extensor exercise (similar to the exercise employed in the present study), which further supports the possibility of LDL/VLDL oxidation following HIE and LIE. In addition, protein carbonyl formation occurs during lipoprotein oxidation (Aldred, 2007), which

may account for the PLS-DA loading of glycoprotein acetyls (CH_3 bonded to a carbonyl) on the HIE and LIE data models.

5.4 Chapter Conclusion

The results of this chapter demonstrate that 3-component PLS-DA modelling of ^{1}H CPMG-edited NMR spectra of intact blood serum reflect subtle metabolic responses to 4 bouts of isokinetic exercise of the knee extensor and flexor muscles at 80% and 40%MVC intensities, for 10 and 20 repetitions, respectively. The results of this study elucidated potential biomolecular markers of glycolysis and gluconeogenesis (lactate and glucose), and lipoprotein oxidation (LDL and VLDL) associated with HIE and LIE. Although these findings are not novel to the study of muscle metabolism, the aforementioned metabolites were detected simultaneously by ^{1}H NMR spectroscopy, alone, as opposed to multiple biochemical assays. However, due to the biomolecular complexity of blood serum, lactate signals were overlapped with resonances of macromolecular components and thus, no numerical values were reported for change in lactate or lipoprotein fatty acids in this study.

The interventions at both intensities appeared to yield similar, subtle metabolic responses since the modality of exercise and total workload (%MVC multiplied by number of repetitions) were standardised. The term ‘subtle metabolic response’ was utilised here since a partial separation was exhibited between classes however, inter-individual variation exceeded the effect of isokinetic exercise. The high degree of inter-individual variation exhibited in the findings of this study was in agreement with other blood plasma/serum NMR-based metabolomic studies (Coolen *et al.*, 2008; Miccheli *et al.*, 2009; Kirwan *et al.*, 2009), despite the use of different exercise modalities (cycling, running and rowing exercise).

Since inter-individual variation exceeded the effect of isokinetic exercise in the present study, prospective metabolomics studies on muscular exercise should consider employing more strenuous interventions to perturb muscle metabolism in an attempt to overcome such variation.

CHAPTER 6: CONCLUSIONS

6.1 Thesis Conclusions

This study investigated the effect of concentric isokinetic exercise of the knee extensor and flexor muscles (functional to standing, walking and running) at 80%MVC and 40%MVC intensities of equal workloads on blood serum, urine and saliva matrices. This study employed a hypothesis-free approach to mathematically determine, by chemometrics, which untargeted metabolites present in multiple biofluid matrices measured by ^{1}H NMR spectroscopy may be associated with muscular exercise of this nature.

The results of chemometric mapping of the ^{1}H NMR spectra of the biofluid matrices collected in the present study demonstrate that metabolic inter-individual variation was greater than the effect of isokinetic exercise of the knee extensors and flexors at 80%MVC and 40%MVC, in terms of score separation. Such variation was attributable to natural differences in metabotypes between the participants at rest and individual responses towards the exercise interventions, despite the implementation of a standardised diet (Appendix 2.5) and controlled exercise conditions. Considering the self-reported exercise history and descriptive characteristics of the participants (Table 2.1), the training background of the participants was quite varied which may account for the inter-individual differences exhibited in the chemometric models. The inclusion criteria were set to a healthy population (absence of injury, illness, and medication) of non-smoking men, aged between 18-40 years, whereas previous exercise studies have recruited a more homogenous population or homogenous subgroups in terms of training background. The results of the present study demonstrate the potential implication for metabolomic assessments to be informative of stress tolerance and individual responses to muscular exercise in a healthy male population, which may be generalised to clinical, active and elite populations when considering exercise prescription in personalised healthcare.

The results of chemometric mapping of the ^{1}H NMR spectral dataset acquired from the saliva matrix elucidated the short-chain organic acids lactate, acetate, *n*-butyrate and formate, and *N*-acetyl sugars as the strongest variable weightings associated with the classifier (change due to effect of isokinetic exercise at both 80%MVC and 40%MVC intensities). Similarly, the salivary concentration of these short-chain organic acids have previously been reported to change in response to Yo-Yo running in 14 Italian, male footballers (Santone *et al*., 2014) however, no semi-quantitative values of these metabolites were reported in this paper to compare with the findings of the present study. However, the origin of these metabolites is equivocal since bacterial metabolism (Silwood *et al.*, 2002) and gland secretion from stimulation via exercise-induced change in the autonomic nervous system (Seals and Victor, 1991) both contribute to the salivary composition.

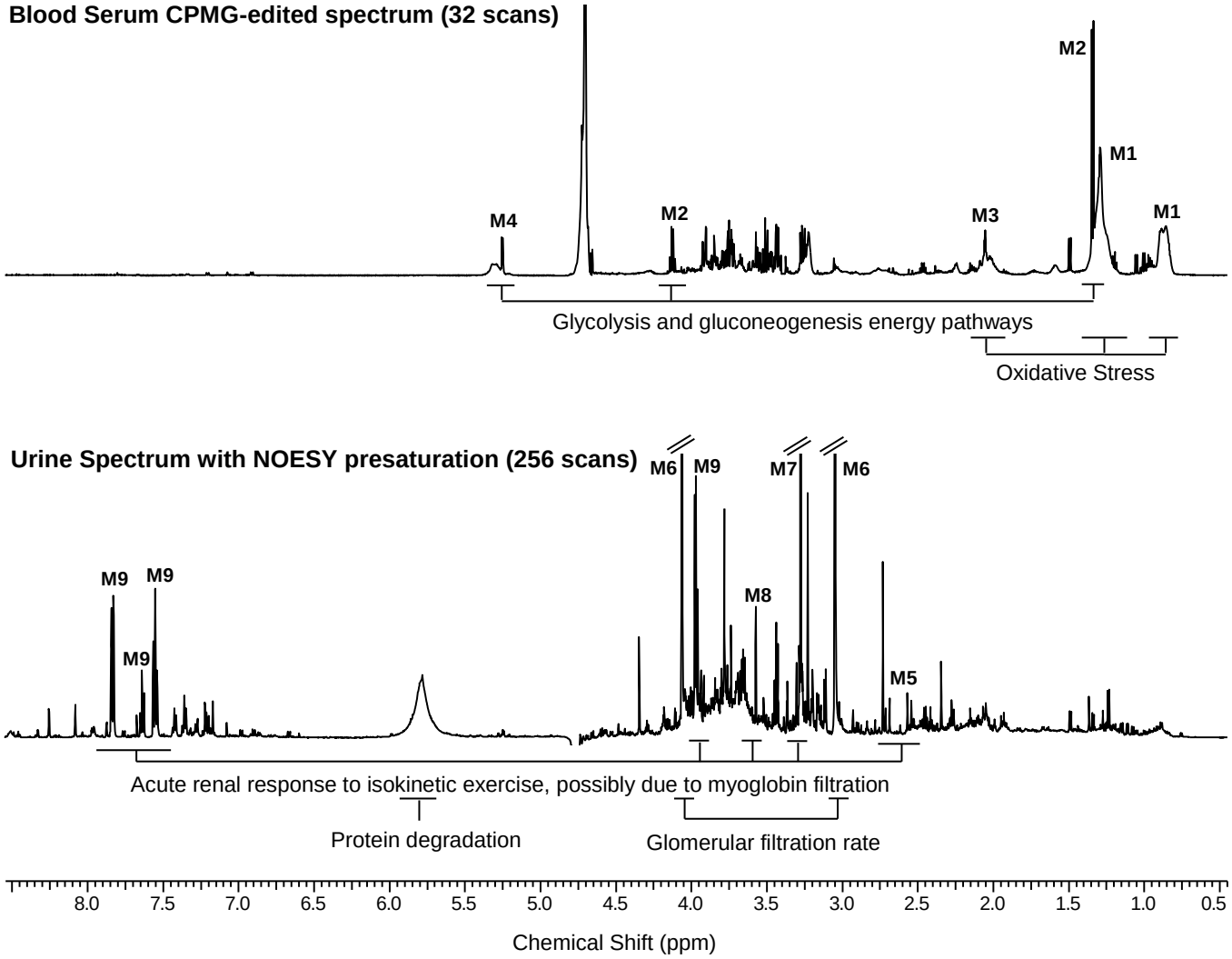
The results of chemometric mapping of the ^{1}H NMR spectral dataset acquired from the blood serum matrix elucidated biomolecular markers of LDL and VLDL oxidation, and glycolysis and gluconeogenesis pathways (lactate and glucose) as the strongest variable weightings associated with the classifier. This suggests that the muscular stress imposed by the isokinetic exercise interventions at both 80%MVC and 40%MVC perturbed the balance of ROS. Nevertheless, blood serum metabolites were less sensitive to change induced by the isokinetic exercise interventions employed in the present study than the urine matrix. A possible reason for this finding is that the blood homoeostasis is maintained by the renal system (Sands and Layton, 2009) and hence, the urine concentration is subject to modifications in response to subtle, systemic physiological change. This finding has implications for sourcing metabolomic information on muscular exercise from non-invasive matrices in prospective studies, which is advantageous for sampling practicality.

The results of chemometric mapping of the ^{1}H NMR spectral dataset acquired from the urine matrix elucidated the low-molecular weight metabolites trimethylamine *N*-oxide, citrate, glycine and hippurate as the strongest variable weightings associated with the classifier. Visual inspection of the spectra revealed an increase in the resonances

assignable to trimethylamine *N*-oxide and a decrease in the resonances assignable to citrate, glycine and hippurate (see Figure 4.9 for illustrative spectra). Although these metabolites are naturally excreted in the urine, this specific change has previously been associated with acute renal injury related to rhabdomyolysis, myoglobinuria caused by server skeletal muscle trauma (Bairaktari *et al.*, 2002), and potential, indirect renal acidosis imposed by sprint running exercise (Pechlivanis *et al.*, 2010). Specifically, excessive urinary excretion of trimethylamine *N*-oxide has previously been associated with renal papilla injury from nephrotoxin insult and may play a role in maintaining osmotic balance (Gartland *et al.*, 1989), whereas marked decreases in hippurate (anion of hippuric acid and derivative of glycine and benzoic acid) and citrate (anion of citric acid in the TCA cycle) may be representative of acute renal tubular injury (Bairaktari *et al.*, 2002).

The results of the present study suggest that concentric muscular loading imposed by isokinetic exercise of the knee extensor and flexors at 80%MVC and 40%MVC may modify metabolites associated with acute renal dysfunction, possibly due to the renal filtration of myoglobin released from stressed muscle into the blood stream (Speranza *et al.*, 2007). This finding potentially contradicts the current knowledge that exercise-induced muscle microtrauma is caused solely by eccentric loading, for reviews see (Nikolaidis *et al.*, 2008; Brancaccio *et al.*, 2010; Brentano and Martins Kruel, 2011) and thus, such new information should be considered to inform exercise prescription. In sport medicine, the urine matrix may provide diagnostic and prognostic information on injured and recovering athletes to determine whether or not they are fit to play, and predict risk of injury based on the pattern exhibited in the biomolecular markers trimethylamine *N*-oxide, citrate, glycine and hippurate.

In general, the high and low intensity isokinetic exercise interventions of equal workloads at 80%MVC and 40%MVC, respectively, both yielded similar metabolic responses, which suggests that a range of exercise intensities can be employed in exercise prescription to elicit such responses provided the workload is standardised (%MVC x number of repetitions). Figure 6.1 summarises the NMR spectroscopy results of this study.



Blood Serum Matrix: Muscular stress imposed by isokinetic exercise at 80%MVC and 40%MVC intensities of equal workloads perturbed the balance of ROS which may have modified the serum content of lipoproteins (M1) and protein carbonyls (M3). Blood serum lactate (M2) increased post-exercise and then reduced 1 hr after exercise cessation, whereas the anomeric glucose signal (M4) exhibited the opposite effect, which is indicative of glycolysis during exercise and gluconeogenesis during recovery. The urinary matrix was more sensitive to exercise-induced change than the serum matrix since the renal system helps maintain blood homeostasis.

Urine Matrix: Due to the acute renal response to isokinetic exercise, an increase in trimethylamine *N*-oxide (M7) was exhibited, together with a decrease in hippurate (M9), citrate (M5) and glycine (M8). This finding was possibly the result of renal filtration of myoglobin proteins relased from the muscle into the blood stream during muscular stress.

Figure 6.1 600 MHz ^{1}H NMR spectra of biofluid matrices obtained from a participant at rest, summarising the functional regions related to isokinetic exercise.

6.2 Limitations

The general limitation of the ^{1}H NMR-based metabolomics approach employed in the present isokinetic exercise study was related to the metabolic complexity of the biofluid matrices, blood serum in particular, which caused signal overlap in various regions of the spectra and thus, various buckets may have contained integrals of more than one signal. However, this limitation was accounted for by chemometric mapping of the metabolomic datasets which elucidated spectral regions assignable to biomolecular components of metabolites that were subject to change following isokinetic exercise. The limitation of the chemometric approach was that the abundant urinary metabolites urea, lactate and creatinine dominated the data models with no class distinction confirmed. Thus, following univariate statistical analysis which revealed no statistically significant difference following isokinetic exercise at either intensity, integrals of these three metabolites were deleted from the entire urinary dataset which resulted in a better discrimination between classes in the refined model and the elucidation of trimethylamine *N*-oxide, hippurate, citrate and glycine integrals.

A limitation of urinalysis in exercise science is that biomarkers have different, and in some cases multiple, biological origins (Sampson *et al.*, 2014) and thus, urinary constituents (pooled) may only serve as indirect biomarkers of muscle metabolism and direct biomarkers of renal function. In addition, paracetamol metabolites were detected in the urine sampled from one individual in this study (Figure 4.2), which may be attributed to the limited information retrievable from the PAR-Q forms (Appendix 2.4) to inform participant inclusion/exclusion, since it specifically questions the use of prescribed medication as opposed to drugs bought over the counter. A further limitation of the health screening form (Appendix 2.4) used in the present study is that the exercise history section is self-reported and only questions the frequency of exercise, discarding fundamental information on exercise intensity, modality and duration.

The saliva matrix is influenced by extraneous factors since whole saliva is a host for bacterial metabolites (e.g. *n*-butyrate, lactate, acetate and formate), gingival crevicular fluid, expectorated bronchial and nasal secretions, derivatives from oral abrasions, viruses and fungi, cellular components and food debris (Kaufman and Lamster, 2002). Since whole saliva is exposed to the oral environment, the use of oral hygiene products may influence the bacterial content of saliva and thus, influence bacterial metabolites present in saliva (Grootveld *et al.*, 2009; Lemanska *et al.*, 2012). The application of sodium azide to all types of biofluid samples to prevent further bacterial metabolism prior to analysis is a routine procedure in ^{1}H NMR spectroscopy-based studies. However, this method does not account for bacterial preponderance and metabolism prior to biofluid collection and thus, the origin of salivary metabolites is equivocal since bacterial metabolism and gland secretion from stimulation via exercise-induced change in the autonomic nervous system both contribute to the salivary composition. In the present study, participants were instructed to rinse their mouths with water prior to saliva collection in an attempt to help remove the aforementioned extraneous factors.

Saliva composition has also been reported to be influenced by the method of collection employed. The salivette collection method is commonplace in exercise science and has the advantage of promoting convenient, hygienic sampling with low risk of spillage. However, the results of this study demonstrate that a ^{1}H NMR spectrum of a water sample exposed to a salivette revealed interference in a number of signals throughout the spectrum (Figure 3.1), exceeding the affected region of δ 3.00-4.00 documented in the literature (Walsh *et al.*, 2006; Bertram *et al.*, 2009). In the present study, the spectral region of δ 3.00-4.50 was deleted from the entire saliva dataset in an attempt to overcome the bulk of salivette interference post-NMR analysis, which consequently resulted in data loss on signals of glucose and CH resonances of amino acids.

6.3 Directions for Future Research

The present study encountered analytical challenges concerning salivette interference on the ^{1}H NMR spectra of saliva and thus, future studies should consider alternative collection methods such as passive drool or expectoration. A further recommendation for future salivary metabolomic studies on exercise is to provide the participants with standardised dental hygiene products (e.g. toothpaste and/or mouth wash) and instructions on usage during the evening and morning prior to saliva collection in an attempt to avoid potential inter-individual variation in salivary microbial content and activity.

Since paracetamol metabolites were detected in the urine sampled from one participant, it necessary to modify the PAR-Q form to consider questioning the use of drugs bought over the counter in addition to prescribed medication prior to urine collection to inform participant inclusion/exclusion in future exercise metabolomics studies. The exercise history section of the health screening form utilised in this study lacks information concerning exercise modality, duration and intensity and thus, prospective exercise metabolomic studies should consider acquiring such necessary information from the participants via a self-reported exercise diary informative of recent physical activity. A detailed training profile of this nature may be informative of exercise tolerance to the intervention employed and may account for inter-individual variation exhibited in chemometric modelling of ^{1}H NMR-based metabolomic datasets. In addition, the information retrievable from a training diary may allow the participants to be categorised into subgroups based on their training status for metabolic comparison.

This study demonstrates the potential implication for the aforementioned biomolecular pattern of urinary trimethylamine *N*-oxide, hippurate, citrate and glycine to reflect renal filtration of muscle proteins from the blood stream (e.g. myoglobin) in response to muscular stress imposed by concentric, isokinetic exercise at 80%MVC and 40%MVC. However, at this stage, the renal response to elevated levels of serum myoglobin following the exercise interventions employed in this study is speculative and thus, further work is

needed to confirm the change in blood serum and urinary myoglobin content following such interventions and enzyme-linked immunoabsorbant assay may be appropriate for this analysis. Since the results of urinary analysis in this study reflect the acute response to four bouts of isokinetic exercise of the knee extensors and flexors at a high and low intensity of a standardised workload, future studies should consider the chronic response to such interventions by profiling the metabolic responses over-time (e.g. weeks).

The importance of exercise participation as a non-pharmacological intervention towards maintaining and improving health has been recognised by the British Government and the Chief Medical Office has made recommendations, publicly accessible, on how much physical activity should be partaken (https://www.gov.uk/government/publications/uk-physical-activity-guidelines). The main focus of this initiative is to reduce the risk of metabolic diseases which are a financial burden on the NHS such as diabetes, hypertension, heart disease and obesity. Since the success of this initiative is dependent on individual assessment and tailored exercise prescription, acquired Government funding may be necessary to develop a metabolomic database in conjunction with other physiological determinants of health and fitness to inform personalised healthcare partly based on metabotypes.

Finally, the metabolic fluctuations in metabolism throughout diurnal cycles may influence exercise performance depending on the time of day of participation due to energy availability and demands. It is routine for elite athletes to engage in physical training both in morning and evening sessions and thus, future research should seek to optimise the training dose specific to the metabolomic profile of an individual at the allocated times of the day.

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APPENDICES

Appendix 1. Poster Presentation Proceedings - Abstract

Proceedings of The Physiological Society

- Human Physiology Abstracts

The Biomedical Basis of Elite Performance (London) (2012) Proc Physol Soc 26, PC47

Poster Communication

Translating systems biology of elite athletes from the laboratory to the sports clinic and arena

Corcoran, O., Lyons, A., Carpenter, R. and Culpan, J.

School of Health, Sport and Bioscience, University of East London, United Kingdom

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In sports science, sports medicine and rehabilitation of sports injury, the nascent fields of exercise 'omics' (metabolomics and proteomics) encompass the identification, characterisation, and quantification of the metabolite and protein content of whole cells, tissues, or body fluids (1). The potential for 'omics' technology holds great promise for talent identification, optimising elite athlete training, avoiding training addiction and overuse injury and policing fair play. Beyond the currently characterised biochemical pathways of cardiac, muscle and kidney function lies the realm of neuroscience interfaced with the psychological aspects of optimised athletic output. Exploring this untapped systems biology hyperspace is limited by current analytical technologies and requires innovative research. This is driven by the ability to identify and quantify novel saliva, sweat, plasma and urine analytes that can function as biomarkers for sculpting elite sports performance. Toxicology evidence of licit enhancers (nutritional supplements and painkillers to mask injury) or illicit enhancers (doping) may simultaneously be monitored (1,2). However, there are many challenges in translating 'omics' from the sports research laboratory to the sporting clinic and arena, and relatively few novel biomarkers have successfully transitioned from discovery to routine use in training. Key barriers to this translation include the range and complexity of the biological samples, a preference for minimally invasive sampling during exercise, the need for "orthogonal" biomarkers (i.e., uncorrelated with existing markers), the presence of high abundance analytes in biological samples that hamper detection of novel low abundance analytes, false positive associations that occur with analysis of high dimensional datasets, lack of routine mobile biosensor devices and the limited understanding of the effects on performance of coaching, differential training regimes, age-related development and performance anxiety. State-of-the-art analytical technologies developed by us (1, 2) focusing on nuclear magnetic resonance (NMR) spectroscopy and associated strategies to overcome these challenges are discussed.

Appendix 2.1 Letter of approval from the University Research Ethics Committee

8 November 2012

Dear Alexander Lyons,

Project Title:	***Application of Metabolomics Techniques to Standardise the Identification of Biomarkers Allied to Exercise, Health and Disease***
Researcher(s):	***Alexander Lyons***
Principal Investigator:	***Roger Carpenter***

I am writing to confirm that the aforementioned study has institutional approval to proceed, following clarification on the proposed research parameters concerning the use of human tissue that were not included in the original application to University Research Ethics Committee (UREC).

In line with the requirements of the Human Tissue Act (2004), UREC has received confirmation from the National Research Ethics Service (NRES) that all human tissue samples must be rendered acellular within seven days, with all remaining cellular material securely destroyed in order that the research be allowed to proceed.

Following written agreement from the research team that the study will adhere to this caveat, UREC is now able to grant approval.

Yours sincerely,

Merlin Harries

University Research Ethics Committee (UREC)
Quality Assurance and Enhancement
Telephone: 0208-223-2009
Email: researchethics@uel.ac.uk

Appendix 2.2 Participant Information Letter

University Research Ethics Committee

If you have any queries regarding the conduct of the programme in which you are being asked to participate, please contact:

Merlin Harries, Quality Assurance and Enhancement (QAE)
External and Strategic Development Service (ESDS)
University of East London, Docklands Campus, London E16 2RD
Email: m.harries@uel.ac.uk

Investigator(s)

Investigator: Alexander Lyons alyons@uel.ac.uk 0208 223 3317
Supervisory Team: Dr. Roger Carpenter r.carpenter@uel.ac.uk; Dr. Olivia Corcoran; Dr. Jane Culpan

Consent to Participate in a Research Study

The purpose of this letter is to provide you with the information that you need to consider in deciding whether or not to participate in this study.

Project Title

PhD Study: Exercise Metabolomics

Project Description

The aim of this project is to measure exercise-induced change in as many metabolites (low-weight molecular products of metabolism) as possible within blood, urine and saliva to elucidate metabolic pathways associated with isokinetic exercise. The exercise will involve isokinetic resistance-based exercise at both high and low intensities, at a standardized workload. This research is being undertaken as part of a Doctor of Philosophy (PhD) award at the University of East London.

Participant Contribution

Non- smoking, healthy, male volunteers, aged 18-40, are invited to take part in this study. If you choose to volunteer for this study, you will be provided with a consent form to sign for participation. Prior to participation, you will be asked to complete a health screening form and physical activity readiness questionnaire to ensure that you are suitable for this research. Volunteers who qualify to participate will be provided with a standardise diet to follow for 24 hours prior to laboratory testing.

Participants are requested to attend three separate laboratory-based isokinetic exercise sessions, 3-7 days apart, which will take place at 9 am. A 10 min warm will be performed, prior to exercise in each session, at 50 W on an exercise bike. Each exercise session consists of isokinetic, concentric muscle contractions of the knee extensor and flexor muscles at a constant velocity of $60^{\circ}.s^{-1}$ separately on both legs using specialised equipment called an isokinetic dynamometer.

The first session will be arranged for both exercise familiarisation and preliminary testing to measure force output (N) of the working muscle groups via five maximal voluntary contractions (MVC). The second and third sessions involve two interventions of equal work (%MVC x number of repetitions), in a randomised order, consisting of high intensity (4 sets of 10 repetitions at 80%MVC) and low intensity exercise (4 sets of 20 repetitions at 40%MVC) to elicit a metabolic response. Samples of venous blood, urine and saliva will be collected pre and post-intervention, and a further blood sample

Appendix 2.2 Continued

will be taken after 1 hr into recovery, for bio-analysis by specialised equipment.

Blood will be collected from a forearm vein in 5 mL vacutainers (Test Tubes) by a qualified person (phlebotomist), in a sanitised environment, in our muscle function testing lab (UH208), and the urine and saliva samples will be collected in a designated facility within our campus. All biofluid samples will be anonymised via a code (for confidentiality) and then the cellular components of these fluids will be removed and disposed of in accordance with the Human Tissue Act 2004. The acellular samples (containing no cells) will then be frozen for storage and kept in our secure labs (UH006/007), until day of analysis.

Potential Hazards, Risks or Discomfort Involved in Participation

Exercise

For most people exercise is safe but can cause discomfort during participation, such as sweating, becoming thirsty, fatigue and increased respiratory rate (heavy breathing). Therefore it is advisable to wear loose comfortable clothing (including shorts) and bring drinking water. Muscle soreness in the thighs may occur after exercise and last up to 48hours, although it is unlikely that this will affect your day-to-day activities.

Blood Sampling

Blood sample collection will be carried out in a sanitized environment by a qualified, experienced person(s) called a phlebotomist. The known risks for this procedure such as cross-infection and/or fainting during blood sampling are well understood and will be minimized with the use of good clinical practice via adherence to health and safety guidelines. Formal risk assessments have been carried out, and a qualified first aid person will be present with access to a telephone.

Aftercare for Participants Post-Testing

The phlebotomist will dress the area of the arm where blood was taken from and advise after care.

Confidentiality of the Data

Information collected from participants will be anonymised (identity removed) and stored on computers with security passwords. Other than the investigators, no other persons will have access. It is our University's policy that after 10years all data will be destroyed using appropriate computer software.

Location

All laboratory sessions will take place in our muscle function testing lab (UH208) at the University of East London – Stratford Campus.

Disclaimer

You are not obliged to take part in this study, and are free to withdraw at any time during tests. Should you choose to withdraw from the programme you may do so without disadvantage to yourself and without any obligation to give a reason.

If you have any further questions please contract Alexander Lyons (details above)

Appendix 2.3 Consent to Participate in an Experimental Programme Involving the Use of Human Participants

Exercise Metabolomics PhD Study

I have the read the information letter relating to the above programme of research in which I have been asked to participate and have been given a copy to keep. The nature and purposes of the research have been explained to me, and I have had the opportunity to discuss the details and ask questions about this information. I understand what is being proposed and the procedures in which I will be involved have been explained to me.

I understand that my involvement in this study, and particular data from this research, will remain strictly confidential. Only the researchers involved in the study will have access to the data. It has been explained to me what will happen once the experimental programme has been completed.

I hereby freely and fully consent to participate in the study which has been fully explained to me, and to publish any anonymised data obtained from my involvement this research. Having given this consent I understand that I have the right to withdraw from the programme at any time without disadvantage to myself and without being obliged to give any reason.

Participant's Name (BLOCK CAPITALS) …….

Participant's Signature ……..

Date: …….

Investigator's Name (BLOCK CAPITALS) ….....…ALEXANDER LYONS…….....……..

Investigator's Signature ……

Date: …….

Appendix 2.4 Physical Activity Readiness Questionnaire (PAR-Q) and Health Screening (adapted from the ACSM)

For most people, it is safe to participate in regular physical exercise and can be beneficial towards health and well-being. This form has been designed as a screening tool to identify individuals who may not be suitable for exercise without clearance from their physician.

Please answer all questions below:

Name (Print): _____ **Date of Birth:** _____

Contact Telephone Number: _____ **Email:** _____

Emergency Contact (Name/Telephone):_____

Has your doctor ever said that you have a heart condition and recommended only medically approved physical activity?	Yes/No
Do you have chest pain brought on by physical activity?	Yes/No
In the past month, have you experienced chest pain at rest?	Yes/No
Do you lose consciousness or lose your balance as a result of dizziness?	Yes/No
Do you have a bone or joint problem that could be aggravated by exercise?	Yes/No
Are you currently being prescribed any medication?	Yes/No
Are you aware of any other reason why you should not participate in exercise?	Yes/No
If so, please give details:	
Has your doctor ever said that you have high blood pressure?	Yes/No
If so, are you currently on medication for your blood pressure?	Yes/No
Has your doctor ever said that you are diabetic?	Yes/No
If so, are you currently on medication for diabetes?	Yes/No
Do you have any serious orthopaedic problems that would affect your ability to exercise? (Joints/muscles/ligaments/tendons/nerves/skin)	Yes/No
Have any of your blood relatives had heart disease, heart surgery or angina?	Yes/No
Do you smoke?	Yes/No
Are you a former smoker?	Yes/No
When was your last physical exam performed by your doctor or nurse?	Yes/No

Exercise History

Little or no exercise	Exercise 1-3 times per week
Exercise 4-5 times a week	Exercise 6 or more times per week

Signature: _____ **Witnessed by:** _____ **Date:** _____

Appendix 2.5 Pre-Test Diet (adapted from Gore, 2000)

		Morning		**Afternoon**	
	Breakfast	Snack	Lunch	Snack	Dinner
24 hours before testing	Cereals (2 c) + skim milk (1 c) Toast (1-2 slices) + jam/honey Fruit juice (1 glass)	1 banana OR 1 cereal bar OR 2 rice cakes + jam/honey	1 roll + ham OR chicken OR tuna OR reduced fat cheese + salad (no butter) 1 carton low-fat fruit yogurt	English muffin (1) + honey (2 tsp) OR Fruit loaf (2 slices)	Chicken (breast, no skin) + rice (2 c) + vegetables OR Pasta (2-3 c) + chicken + tomato sauce + vegetables
Testing day (7 am meal)	2-3 toast + honey Fruit juice				

Abbreviations: c = cups, tsp = teaspoons

To meet fluid requirements you must drink 50-60 mL of fluid/kg body weight – equivalent to 3-4 L of water per day.

If you do not wish to follow precisely the diet offered here, you should ensure that any substitutions are nutritionally equivalent and report details to researcher.

Appendix 2.6 Results of Tests of Normality for Targeted Urinary Variables using SPSS

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
HIEpreCr	.215	14	.079	.886	14	.072
HIEpostCr	.396	14	.000	.568	14	.000
HIEpreUr	.221	14	.062	.749	14	.001
HIEpostUr	.390	14	.000	.463	14	.000
HIEpreLa	.308	14	.001	.602	14	.000
HIEpostLa	.380	14	.000	.626	14	.000
LIEpreCr	.182	14	.200*	.909	14	.151
LIEpostCr	.327	14	.000	.589	14	.000
LIEpreUr	.275	14	.005	.851	14	.023
LIEpostUr	.427	14	.000	.457	14	.000
LIEpreLa	.377	14	.000	.628	14	.000
LIEpostLa	.401	14	.000	.402	14	.000
La.Cr_preHIE	.190	14	.183	.885	14	.069
La.Cr_postHIE	.459	14	.000	.362	14	.000
La.Cr_preLIE	.267	14	.008	.693	14	.000
La.Cr_postLIE	.439	14	.000	.386	14	.000
HIE_preUr.Cr	.104	14	.200*	.966	14	.823
HIE_postUr.Cr	.125	14	.200*	.925	14	.257
LIE_preUr.Cr	.218	14	.071	.925	14	.261
LIE_postUr.Cr	.158	14	.200*	.948	14	.525

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

La Lactate/TSP; **Ur** Urea/TSP; **Cr** Creatinine/TSP; **Ur.Cr** Urea/Creatinine; **La.Cr** Lactate/Creatinine

Appendix 2.7 Results of Paired Samples *t*-Tests for Targeted Urinary Variables using SPSS

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	HIEpreCr - HIEpostCr	-25.76018	82.71131	22.10553	-73.51626	21.99591	-1.165	13	.265
Pair 1	LIEpreCr - LIEpostCr	-8.83871	43.92514	11.73949	-34.20032	16.52291	-.753	13	.465
Pair 1	HIE_preUr.Cr - HIE_postUr.Cr	-.21887	.91049	.24334	-.74457	.30683	-.899	13	.385
Pair 1	LIE_preUr.Cr - LIE_postUr.Cr	-.41992	.98226	.26252	-.98706	.14722	-1.600	13	.134
Pair 1	La.Cr_preHIE - La.Cr_postHIE	-.15942	.49313	.13179	-.44414	.12531	-1.210	13	.248

La Lactate/TSP; **Ur** Urea/TSP; **Cr** Creatinine/TSP; **Ur.Cr** Urea/Creatinine; **La.Cr** Lactate/Creatinine

Appendix 2.8 Results of Wilcoxon Signed Rank Tests for Targeted Urinary Variables using SPSS

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The median of differences between HIEpreUr and HIEpostUr equals 0.	Related-Samples Wilcoxon Signed Rank Test	.875	Retain the null hypothesis.

	Null Hypothesis	Test	Sig.	Decision
1	The median of differences between LIEpreUr and LIEpostUr equals 0.	Related-Samples Wilcoxon Signed Rank Test	.594	Retain the null hypothesis.

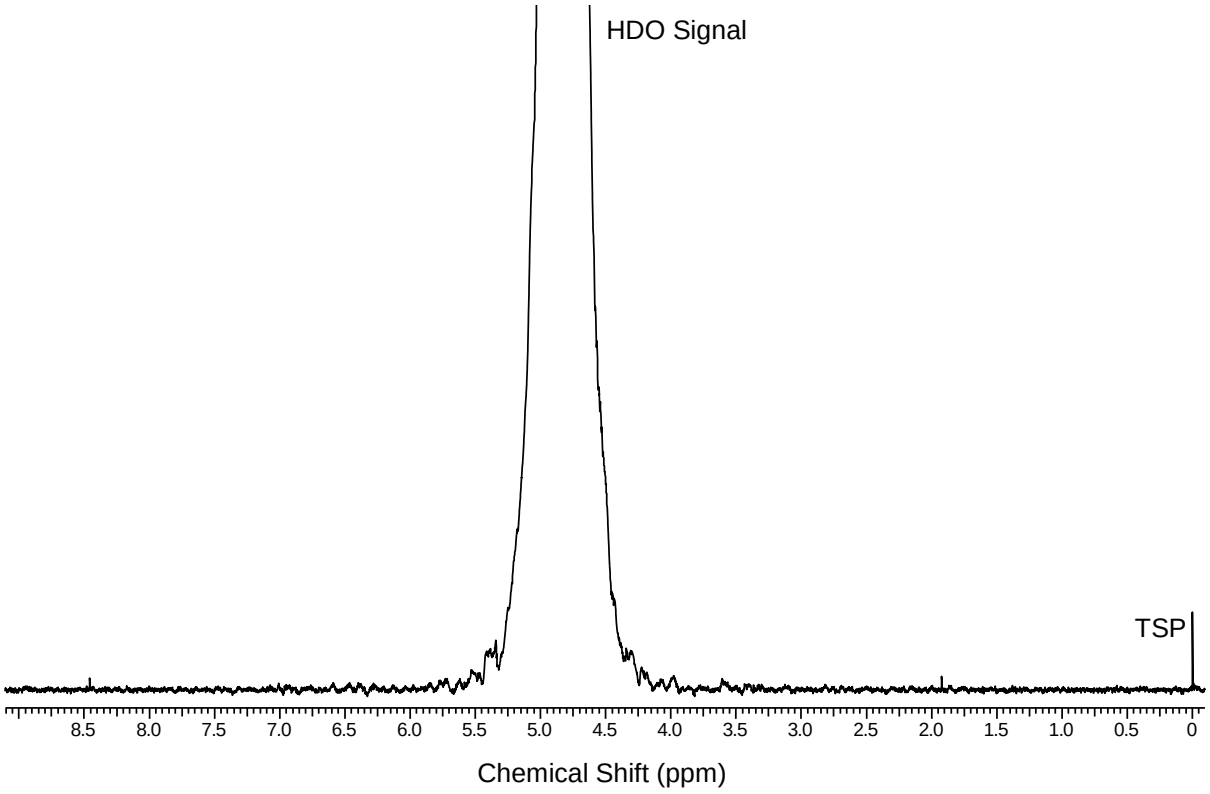
	Null Hypothesis	Test	Sig.	Decision
1	The median of differences between HIEpreLa and HIEpostLa equals 0.	Related-Samples Wilcoxon Signed Rank Test	.074	Retain the null hypothesis.

	Null Hypothesis	Test	Sig.	Decision
1	The median of differences between LIEpreLa and LIEpostLa equals 0.	Related-Samples Wilcoxon Signed Rank Test	.245	Retain the null hypothesis.

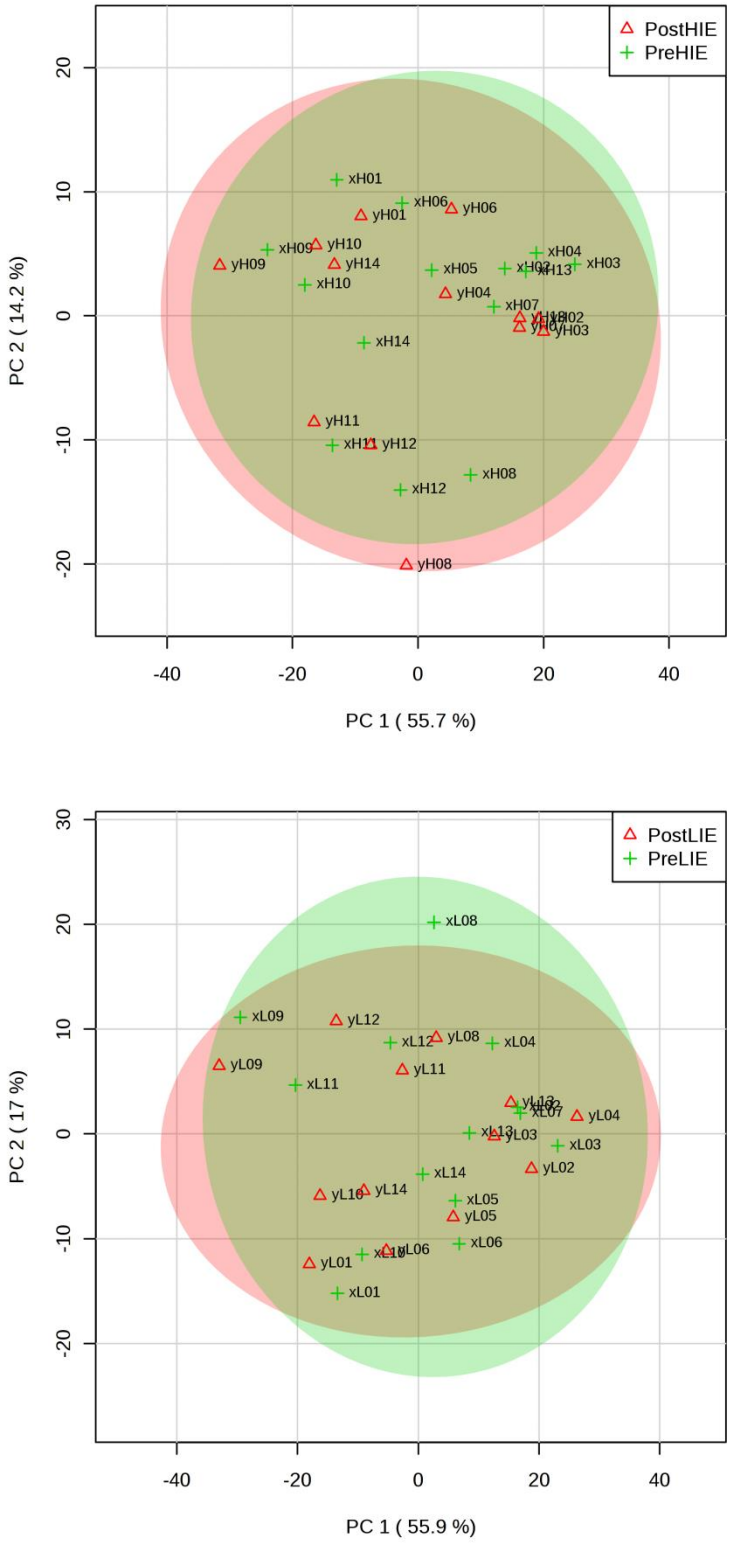
	Null Hypothesis	Test	Sig.	Decision
1	The median of differences between La.Cr_preLIE and La.Cr_postLIE equals 0.	Related-Samples Wilcoxon Signed Rank Test	.272	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

La Lactate/TSP; **Ur** Urea/TSP; **Cr** Creatinine/TSP; **Ur.Cr** Urea/Creatinine; **La.Cr** Lactate/Creatinine



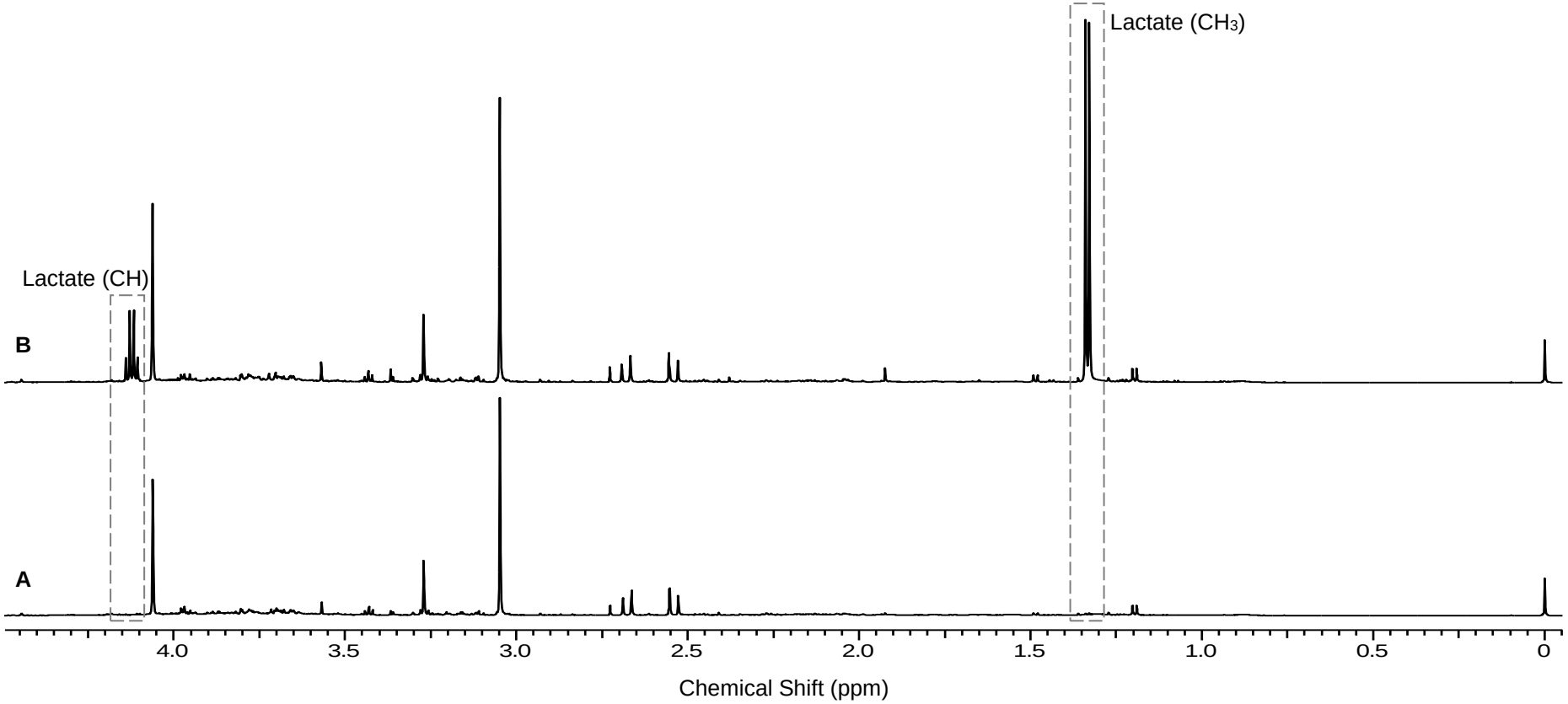
Appendix 3.1 400 MHz ^{1}H NMR ZG spectrum (8 scans) of water exposed to a salivette and treated with TSP (internal standard for the chemical shift reference). No pronounced artefacts were present in this spectrum, thus indicating no major interference from electronic noise from the NMR instrument.



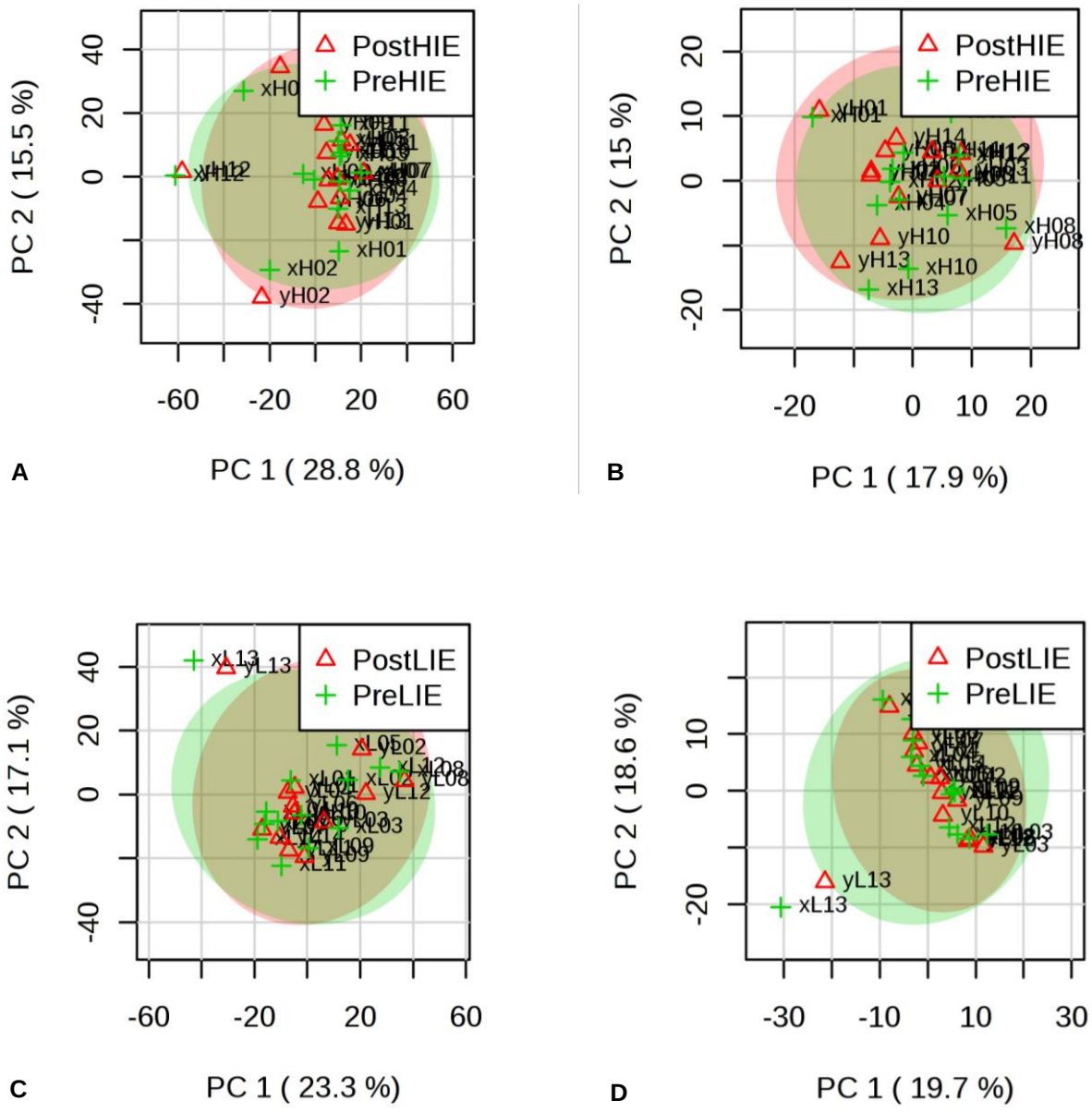
Appendix 3.2 PCA scores plot of 600 MHz ^{1}H NMR spectra of saliva (excluding the region of δ 3.00-4.50), normalised to sum of intensities, taken from 14 healthy men. **(A)** HIE dataset and **(B)** LIE dataset. All scores fell within Hotelling's 95% confidence ellipses, hence no outlying scores.

Appendix 4.1 Urinary pH (shaded) and Osmolality (mOsmo.kg H_2O) for each sample collected

Participant	Pre-HIE	Post-HIE	Pre-LIE	Post-LIE	Pre-HIE	Post-HIE	Pre-LIE	Post-LIE
01	5.18	5.32	5.32	5.15	935	1551	1727	911
02	5.95	5.4	6.79	6.37	730	301	1029	86
03	6.71	6.41	6.86	7.00	112	197	982	951
04	5.27	5.44	5.28	5.19	809	510	744	767
05	8.3	7.9	5.27	4.99	849	750	823	691
06	5.2	5.14	5.76	6.09	768	791	807	771
07	5.25	5.26	5.49	5.32	889	899	980	891
08	5.46	5.68	7.2	7.18	1637	80	492	336
09	5.63	5.69	5.61	6.52	246	153	350	121
10	6.7	5.6	6.56	6.5	94	565	246	277
11	5.67	5.86	7.46	7.32	392	626	618	762
12	7.18	7.81	7.5	7.56	778	481	500	170
13	5.45	5.43	5.7	5.57	1115	524	1136	358
14	6.1	6.7	6.32	6.89	684	931	340	482



Appendix 4.2 600 MHz ^{1}H NMR spectra (256 scans) of urine obtained **(A)** pre- and **(B)** post-HIE from participant 11. An increase in urinary lactate signals (δ 3.05 and 4.13) following exercise was exhibited in the spectra from all participants, yet the increase was more pronounced for participant 11. The LIE results of urine were in agreement with this finding.



Appendix 4.3 PCA Models (Scores Plots) generated from the urinary spectral datasets (HIE and LIE) to determine the effects of normalisation to either the creatinine signal (δ 3.05) or to sum of intensities on the percentage of variance explained. **(A)** HIE, normalised to creatinine. **(B)** HIE, normalised to sum of intensities. **(C)** LIE, normalised to creatinine. **(D)** LIE, normalised to sum of intensities. Data normalisation to creatinine provides the most robust model in terms of percentage of variance explained.