Detection and measurement of stress hormones with mass spectrometry

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DECLARATION

I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any degree other than that of Doctor of Philosophy being studied at the University of Greenwich. I also declare that the work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others.

Signed ……

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Date ……

Signed ..... Date ……

Dr Birthe Nielsen

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Dr Judith Allgrove

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ABSTRACT

The purpose of this thesis was to develop and validate a liquid chromatography mass spectrometry (LC-MS) method to measure salivary cortisol and testosterone. Furthermore, to use this method to analyse saliva samples collected during an exercise study measuring the hormonal response to acute exercise. The ongoing contribution to knowledge provided by this research is the application of a developed and validated LC-MS method to analysis of cortisol from pre and post exercise saliva samples, and evaluation of the cortisol and testosterone response to three different training sessions; including an interval session (INT), tempo run (TEMP) and aerobic circuit training (CIR). An LC-MS method was developed to measure cortisol and testosterone. The mass spectrometer used was a triple quadrupole BioQ (Waters/Micromass, UK) coupled with a 1200 series HPLC machine (Agilent Technologies, UK). Optimisation of various parameters was undertaken including: cone voltage and capillary voltage, followed by optimisation of liquid chromatography (LC) parameters such as; mobile phase gradient and flow rate. Saliva samples were collected at rest, and hormones measured with the new LC-MS method and ELISA to validate the developed method. Finally, heart rate and salivary and plasma cortisol and testosterone response to acute exercise were tested, to compare the response to different training protocols in runners. Following this, the MS method underwent further validation against ELISA. Results revealed optimum MS parameters were: cone voltage 25 V; capillary voltage 3.5 kV and LM and HM resolution 8 Da. For the LC method, mobile phase flow rate was optimised at 0.1 mL/min, with a gradient profile ranging from 50 to 95% methanol (MeOH), and a run time of 15 minutes. Sample preparation was also considered (solid phase extraction); and wash phase of 10% MeOH, 10 times sample concentration and reconstitution with 80% MeOH were optimised to improve analyte recovery and detection. Comparison of cortisol measured at rest with LC-MS and ELISA ($n=22$) revealed a correlation between methods ($r=0.83$, $P<0.001$). The exercise study showed the INT elicited a higher peak heart rate (172±11) than CIR (148±10) or TEMP trials (163±10). INT also produced a higher RPE (15 ±2) than CIR (13±1) and TEMP (14±2) trials. Salivary cortisol increased at all time points post exercise in INT and remained higher than rest (1.29 ± 0.51 ng/mL) at 60 minutes recovery (3.13 ±1.59 ng/mL). Salivary testosterone increased pre to post exercise in CIR (119.7±39.5 pg/mL to 150.1 ±51.5 pg/mL), TEMP (142.4±76.5 pg/mL to 185.0±49.1 pg/mL) and INT (117.5±23.5 pg/mL to 176.3±46.0 pg/mL) trials. There was also a correlation between cortisol

measured in plasma and saliva (r=0.813, p=0.0001). Additionally, comparison of salivary cortisol measurements obtained with mass spectrometry and ELISA (n=85) showed a correlation (r=0.934, p=00001). In conclusion, validation revealed cortisol could be quantfied accurately and precisely with LC-MS. Additionally, INT elicited a higher mean RPE and cortisol response than TEMP or CIR.

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LIST OF ABBREVIATIONS

ATCH	Adrenocorticotrphic hormone
ARH	Adrenocorticotrphic releasing hormone
ANOVA	Analysis of variance
BSTFA	N,O-Bis(trimethylsily)trifluoroacetamide
CAR	Cortisol awakening response
CIR	Circuit session
CRH	Corticotropin releasing hormone
CRM	Charged residue model
C/T	Cortisol/testosterone
CV	Coefficient of variation
Da	Daltons
DAP	2, 6-diaminopyridine
DC	Direct current
DTE	1,4-dithioerythritol/trimethyliodosilane
EI	Electron ionisation
ELISA	Enzyme linked immunosortbent assay
ESI	Electrospray ionisation
FSH	Follicle stimulating hormone
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
GnRH	Gonadotropin releasing hormone
h	Hour
HETP	Height equivalent of theoretical plate
HM	High mass
HMP	2-hydrazino-1-methylpyradine
HPA axis	Hypothalamic-pituitary-adrenal-axis
HPLC	High performance liquid chromatography
HR	Heart rate
ID	Internal diameter
IEM	Ion evaporation model
INT	Interval session
IS	Internal standard
IT	Interval training
K_2EDTA	Ethylene diamine tetraacetic acid

kg	Kilograms
km	Kilometres
kV	Kilovolts
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LH	Lutenising hormone
LM	Low mass
LOD	Limit of detection
LOH	Late onset hypogonadism
LOQ	Limit of quantification
m	Metre
MeCN	Acetonitrile
MeOH	Methanol
min	minute
mL	Mililitre
mmHg	Millimetres of Mercury
MNBAn	2-methyl-6-nitrobenzoic anhydride
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSTFA	Methyl-N-(trimethylsilyl) trifluoroacetamide
MTBE	Methyl-*tert*-butylether
m/z	Mass to charge ratio
NFO	Non functioning overreaching
ng	Nanogram
PFP	Pentaflurophenyl
pg	Picogram
pKa	Acid dissociation constant
RIA	Radioimmunoassay
RF	Radio frequency
RF/DC	Radio frequency/direct current ratio
RM	Repetition maximum
RPE	Rating of perceived exertion
RPM	Revolutions per minute
SD	Standard deviation
SEM	Standard error of the mean

S/N	Signal to noise
SNS	Sympathetic nervous system
SPE	Solid phase extraction
SRM	Selected reaction monitoring
T/C	Testosterone/Cortisol
TEMP	Tempo run
TMSI	Trimethylsilylimidazole
TFA	Trifluroacetic acid
THF	Tetrahydrofuran
TR	Tempo run
UPLC	Ultra performance liquid chromatography
V	Volts
VO_{2max}	Maximal oxygen uptake
µg	Micrograms
^{o}C	Degrees celcius

1. INTRODUCTION

Acute stress during an exercise intervention can be assessed by a change in the concentration of the steroid hormones cortisol and testosterone. The acute hormonal response to exercise has been reported with different exercise modes, intensities and duration and recovery also considered. The ratio between cortisol and testosterone has also been suggested as a marker of overtraining syndrome in athletes (Aldercreutz *et al.*, 1986). Quantification can be from blood, urine and saliva; the latter a non invasive alternative to blood for hormone measurement. Until recently immunoassays were the main technique used to quantify cortisol and testosterone; however liquid chromatography tandem mass spectrometry has become the gold standard for quantification of small molecules in pharmaceutical, environmental and clinical applications and has potential to offer rapid, accurate analysis in exercise research.

Liquid chromatography-mass spectrometry (LC-MS) faces ongoing challenges for faster chromatography and detection of lower concentrations of analytes. Saliva matrix effects can limit ionisation efficiency, and given the low level of testosterone in saliva it is important to maximise sensitivity whilst maintaining high resolution; therefore sample preparation is important. Method validation is also important to achieve acceptance that the method can be used to analyse saliva samples effectively (ICH, 1996). This research project is multifaceted and firstly aims to assess the validity of mass spectrometry as a tool to measure cortisol and testosterone in saliva. In extension, to consider the limited use of LC-MS in sport and exercise science; saliva samples collected before and after acute exercise are analysed with this technique. Addressing the need for further analysis of the role of cortisol and testosterone in the exercise stress response this work also endeavours to investigate the effect of acute training protocols on hormone secretion.

1.1 Stress

Stress can be psychological or physical and both types can be acute or chronic. Psychological stress is referred to as events or situations that challenge a person's psychological and/or physiological homeostasis (Cannon, 1935). Lazarus and Cohen (1977) identified three categories of stress including; cataclysmic events which include

natural disasters and war; personal stressors, which are negative life events including death, divorce or loss of job and absence of positive life events, and thirdly; daily hassles which are chronic background stressors, for example work environment. Not everyone will experience events with the same level of stress, and to experience an event or situation as stressful it has to be perceived or appraised as such (Lazarus, 1966). Physical stress includes environmental stressors such as extreme cold, heat and exercise stress. Exercise can encompass a short bout i.e. under an hour of cardiovascular exercise, a weight training session or a longer endurance effort such as a marathon. The time it takes for the body to return to 'normal' and reach homeostasis depends on the severity of the stress.

The hypothalamus is activated after the onset of a stressor and secretes corticotrophic releasing hormone (CRH), which stimulates the pituitary gland to secrete adrenocorticotrophic hormone (ACTH). This hormone in turn stimulates the adrenal glands to release stress hormones such as adrenaline and cortisol. The effects of a stressor reflect the bodies 'fight or flight' response (acute biological response to stress) and are characterised by an increase in heart rate, blood pressure and respiratory rate, breakdown of glycogen stores to mobilise glucose and a suppressed immune system (Axelrod and Reisine, 1984). This is usually a short term reaction as the stressor which the body responds to then returns to 'normal'. Activation of the sympathetic nervous system (SNS) leads to the short term release of catecholamines into the circulation (adrenaline and noadrenaline), followed by the release of cortisol which is slower and can be more prolonged. Acute stress has also been shown to increase circulating levels of testosterone (Sutton *et al.,* 1973) and activation of catecholamines may be one of the main mechanisms stimulating secretion of testosterone during stress. It is known that noradrenaline stimulates production of gonadotropin-releasing hormone (GnRH) and lutenising hormones (LH), both in men and in women (Chrousos, 1998). However, the exact mechanisms for the increase in testosterone are under investigation.

Chronic stress can occur if the stressor is not removed and leads to elevated corticosteroids which can have long term health implications such as headache, anxiety disorder, mood swings, memory disturbances, depression, anxiety and loss of concentration (Carroll, 1992). Long term physical stress has also been shown to elicit a decrease in circulating testosterone (Franca *et al.,* 2006), and androgens are thought to play a role in carbohydrate metabolism. Androgens may have a suppressive effect on hepatic gluconeogenesis

(Tarnopolsky *et al*., 1990). There is also evidence that tesotosterone may increase lypolysis and therefore offer a glucose sparing function, as studies of late pubertal boys revealed decreased lean mass and increased adiposity in those who were testosterone deficient; with decreased lypolysis and free fatty acid mobilisation was suggested as the cause (Mauras *et al*., 1987). In sport chronic high stress levels with limited recovery have been suggested as an indicator of overtraining status or unexplained, underperformance syndrome (Urhausen, 2002). Stress biomarkers are commonly measured in blood plasma and serum, and also urine and saliva and this work focuses on the response of cortisol and testosterone.

1.2 Stress biomarkers

1.2.1 Cortisol

Cortisol ($C_{21}H_{30}O_5$) is a glucocorticoid produced by the cells of the adrenal cortex (for structure see figure 1.01). Cortisol represents a chemical response of the body to stress and also plays a major role in the mobilisation of glucose into the blood stream. This is accomplished through inhibition of insulin production and sensitivity during SNS activity (Braunwald *et al.*, 2001), and by breakdown of stored protein into glucose (Juhan, 1998). The hormone stimulates gluconeogensis and mobilisation of free fatty acids to make more glucose available (Salway, 2006). Additionally, cortisol also has anti-inflammatory effects and acts on body defence mechanisms to suppress tissue response to injury (Brook and Marshall, 2001).

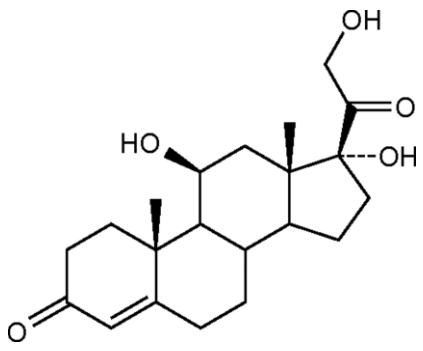


Fig 1.01 Chemical structure of cortisol (Dvorak *et al*., 2006)

Normal production of cortisol by cells of the adrenal cortex is dependent on two messengers in a chain called the hypothalamic-pituitary-adrenal axis (HPA axis). When stress is experienced, the hypothalamus releases the messenger corticotropin releasing

hormone (CRH). CRH is transported to the anterior pituitary and signals specialised cells to release ACTH into the bloodstream. ACTH is transported to the adrenal glands where it signals cells of the *zona fasciculata* and *reticularis* layers of the adrenal cortex. These cells increase production of cortisol and androgens (Brook and Marshall, 2001).

1.2.2 Testosterone

Testosterone ($C_{19}H_{28}O_2$) (figure 1.02) is an androgen produced by the adrenal gland, the testis in males and the ovaries in females. Release of testosterone is controlled by the hypothalamus and it regulates human growth and reproduction, including contribution to the development of the male genitals. Additionally, its anabolic actions also build skeletal and muscle tissue which is juxtaposed to the catabolic actions of cortisol (Norman and Litwack, 1997). During stress testosterone production is thought to decrease as it is inhibited by the release of cortisol, this is thought to reflect the effect of cortisol on testosterone production in the testes (not production by the central regulatory components i.e. HPA) (Cummings *et al.*, 1983). Additionally, it is hypothesised that steroidogentic enzymatic activity for testosterone synthesis within the testes is disrupted (Castro and Matt, 1997). Production of testosterone is mainly from the male gonads; therefore, circulating levels in women are lower compared to men, with women's ovaries and adrenal glands sharing the responsibility for testosterone production (typical resting hormone levels, table 1.1). Testosterone regulation is controlled by release of GnRH in the hypothalamus. This activates the release of LH and follicle stimulating hormone (FSH). LH triggers production of testosterone from cholesterol in the Leydig cells and FSH stimulates secretion of androgens and promotes spermatogenesis in males. If levels are high, testosterone can suppress production of GnRH in turn reducing production of FSH and LH (Brook and Marshall, 2001).

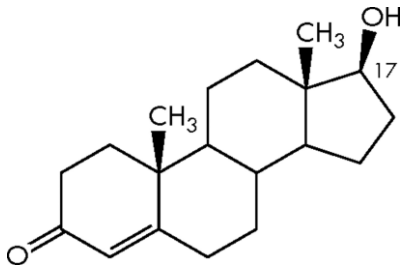


Fig 1.02 Chemical structure of testosterone (Saudan *et al*., 2006)

Table 1.1 Typical concentration of cortisol (Institute of Isotopes, 2011) and testosterone (Medlineplus, 2010a) in blood**; cortisol** (Medlineplus, 2010b) and testosterone (Bao *et al*., 2008) in urine and cortisol (Aardal and Holm, 1995) and testosterone (Dabbs, 1990) in saliva.

	Cortisol	Testosterone	
		Men	Women
Blood	53.4 – 264 ng/mL	3.0-10.8 ng/mL	0.3-0.95 ng/mL
Urine	10,000-100,000 ng/24 hr	64000-374,000 ng/24 hr	14200-107,000 ngl/24 hr
Saliva	1-8 ng/mL	0.049-0.189 ng/mL	0.006-0.044 ng/mL

1.2.3 Hormone biosynthesis

Steroid hormones are synthesised from cholesterol through a series of enzyme controlled reactions (Miller, 1988) (figure 1.03). Cortisol is produced in the adrenal cortex, cholesterol is converted to pregnenolone with cholesterol side chain cleavage enzyme and then this in turn is converted to 17a-OH-pregnenolone with a reaction of 17a-hydroxylase/17-20-lyase. 17a-OH-pregnenolone forms 17a-OH-progesterone produced by the enzyme 3b-hydroxysteroid dehydrogenase/D5-D4-isomerase, following this 17a-OH-progesterone is converted to deoxycortsol to cortisol with the enzyme 21-hydroxylase and the final stage conversion from deoxycortisol with enzyme 11b-hydroxylase. Testosterone can be formed in the testes (male) or ovaries (female) by various pathways, however the principal pathway suggests identical reactions to cortisol until 17a-OH-progesterone followed by conversion to androstenedione with 17a-hydroxylase/17-20-lyase and finally testosterone by enzyme 17b-hydroxysteroid dehydrogenase (Norman and Litwack, 1987). Biosynthesis occurs in the mitchondria and smooth endoplasmic reticulum of cells (Paynes and Hales, 2004). As previously mentioned, when stress occurs, release of steroid hormones is controlled by the hypothalamus. The physiological effect of steroids is initiated when they enter target cells and bind to receptors which act as transcriptional activators of steroid response genes. Furthermore, hormone release exhibits a natural daily variation.

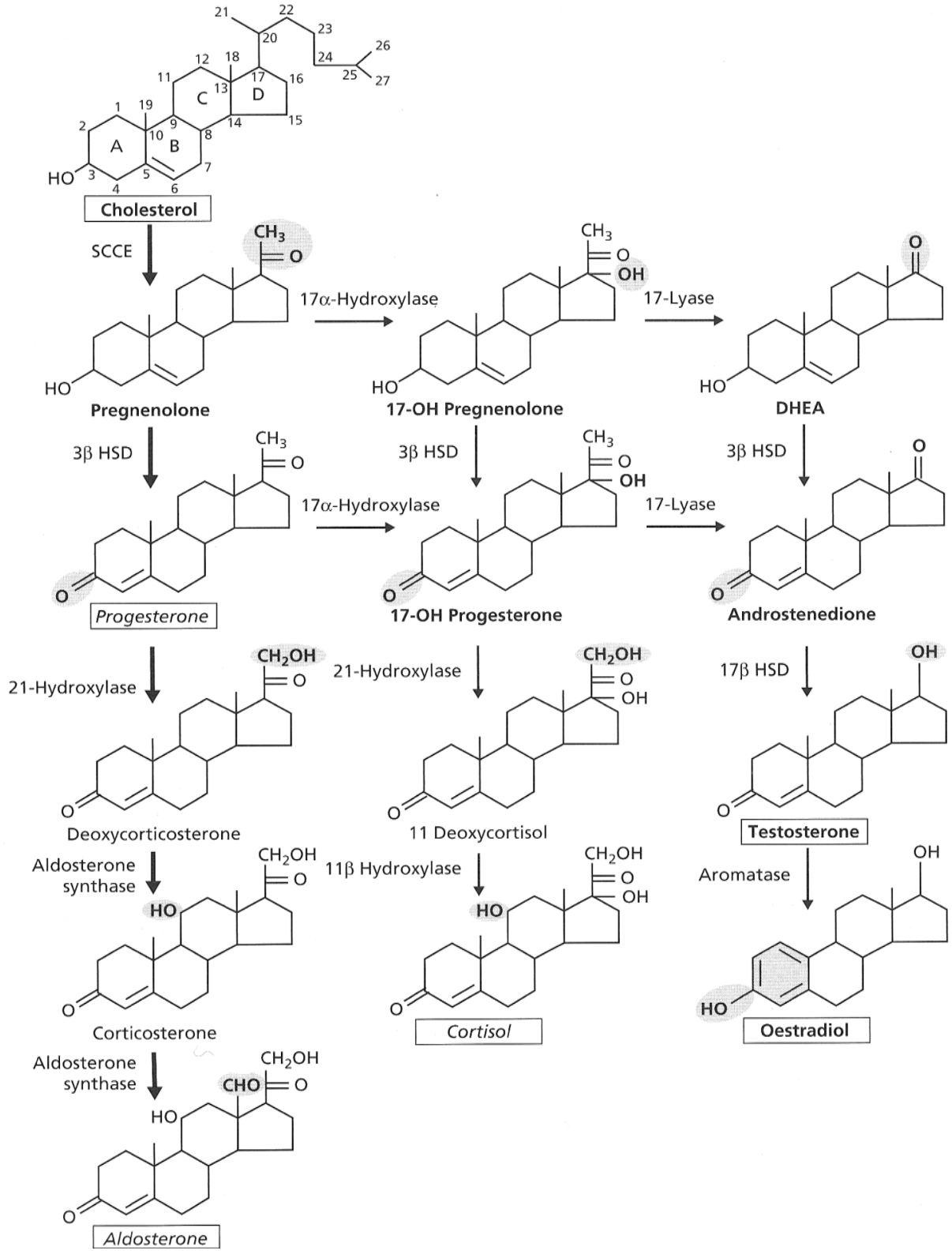


Figure 1.03 Biological synthesis pathways of hormones (adapted from Brook and Marshall, 2001, p69)

1.2.4 Biological variation

Cortisol and testosterone exhibit a natural diurnal variation in addition to gender differences. Concentrations of these hormones also vary depending on the biological matrix examined (table 1.1), however there is evidence that correlations exist between blood and salivary measures for both cortisol (Kirschbaum and Hellhammer, 2000) and testosterone (Vittek, 1985).

Diurnal variation

It is established that cortisol exhibited a diurnal variation and may be secreted episodically (Hellman *et al.,* 1970; Krieger *et al.,* 1971). Levels increase on awakening and peak after 45 minutes then decrease throughout the day (figure 1.04). The increase of cortisol upon awakening is known as the cortisol awakening response (CAR). Reports have suggested the CAR can be affected by gender with evidence that women elicit a higher response than men (Pruessner *et al.,* 1997; Clow *et al.,* 2004; Weekes *et al.,* 2008). Similarly, chronic psychosomatic stress has been found to intensify the CAR (Wust *et al.,* 2000, Schulz *et al.,* 1998). Contrastingly, there is evidence that taking oral contraceptives has a tendency to attenuate the CAR (Pruessner *et al.,* 1997; 1999), therefore when measuring cortisol it is important to control for these variables.

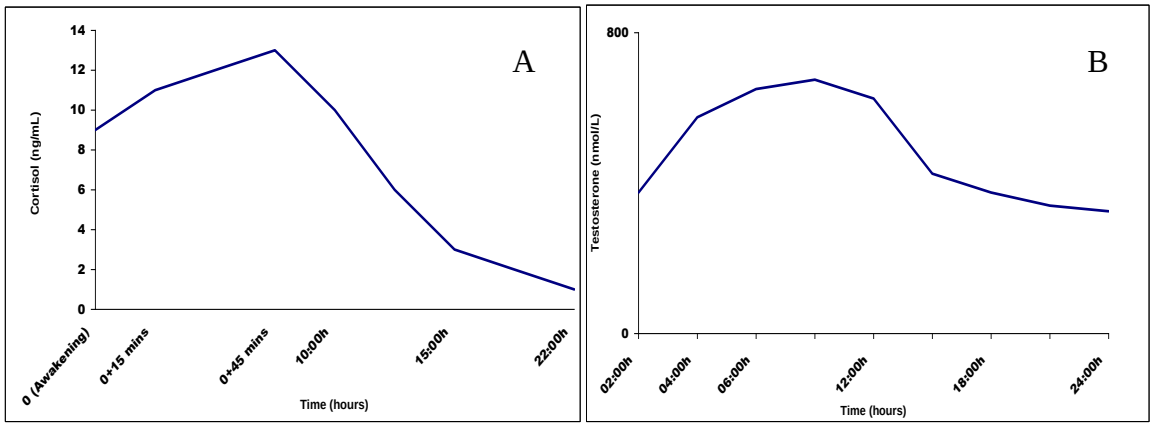


Figure 1.04 Diurnal variation of salivary cortisol (A) adapted from Harris *et al*. (2010); serum testosterone (B) adapted from Hong (2008)

Previous research has illustrated there are also daily variations in testosterone with the highest concentration observed in the morning between 5.30 am and 8.00 am and the lowest approximately 12 hours later (Resko and Nes, 1966; Fairman and Winter, 1971; Baxendale *et al.,* 1980) (Figure 1.04B). It is suggested that the morning testosterone peak

is due to increased synthesis (Diver *et al.,* 2003); however the mechanisms for this variation are still not fully understood (Walton *et al.,* 2007).

Testosterone changes with age have also been considered and there is evidence for a decline with age (Vermeulen *et al*., 1972; Bremner *et al*., 1983; Korenman *et al*., 1990; Simon *et al*., 1992; Tennekoon & Karunanayake, 1993; Morley *et al.,* 1997; Harman *et al.,* 2001), however Diver *et al.,* (2003) suggest that fit healthy men over 60 years can achieve levels of circulating testosterone in the concentration range of young men while maintaining a circadian rhythm. This is supported by Szulc *et al.,* (2001) and Khosla *et al.,* (2001). Diver *et al.,* (2003) studied the effect of age on diurnal variation in young and middle aged men and reported that diurnal variation was still visible in the older men and was not affected by lower resting levels typically observed in this population. These studies show that fit healthy men may not show an age related decline in testosterone; however other studies suggest health issues may contribute to a blunting of the diurnal rhythm and potential decline in testosterone in some older men.

Correlation between biological matrices

Numerous studies have assessed the correlation of hormone levels between biological matrices. Cortisol is a small, highly lipid soluble molecule and can pass easily through the lipid-bilayer membranes of nucleated cells. However, acute levels of free cortisol in saliva are lower due to a relative abundance of the cortisol-metabolising enzyme 11-β hydroxysteroid dehydrogenase (Kirschbaum and Hellhammer, 2000). Salivary levels are around 1-2% of total cortisol in the lower range and 8-9% in the upper range (Hellhammer *et al.,* 2009), and levels in saliva correlate with free cortisol in the blood (Kirschbaum and Hellhammer 1994; Paccotti *et al.,* 2005; Kirschbaum and Hellhammer, 2000; Lac *et al.,* 1993; Kirschbaum *et al.,* 1999). This high agreement is due to cortisol entering saliva by passive diffusion, independently of saliva flow rate. This is in contrast to other components found in saliva such as immunoglobulin A, where concentrations in saliva are affected by secretion rate from the saliva gland (Kirschbaum and Hellhammer, 2000). Despite the correlation there is a lag time for cortisol after acute stress (including exercise) with maximal levels recorded after 10-30 minutes (Kirschbaum and Hellhammer, 1989, 2000; Heinrichs *et al.,* 2001; Hough *et al.,* 2011). The diffusion from plasma to saliva occurs quickly, after intravenous injection cortisol was evident in saliva after less than one minute and peak levels lagged only 2-3 minutes after blood (Kirschbaum and Hellhammer,

2000). However, following acute exercise Hough *et al*., (2011) revealed plasma cortisol concentration peaked 10-20 minutes post exercise and saliva 10 minutes later suggesting a longer lag time than pharmaceutical interventions.

Similarly to cortisol, testosterone passively diffuses into the saliva and free testosterone in saliva also appears to be correlated with serum measures. Vittek *et al.,* (1985) and Lac *et al.,* (1993) showed a significant correlation between salivary and serum free testosterone (r=0.97). After exogenous testosterone administration, Wang *et al.,* (1981) found serum and salivary testosterone rose abruptly and in parallel, suggesting there is no lag time. As with cortisol there appears to be a lag time for peak tesotosterone in plasma and saliva; Hough *et al.,* (2011) showed peak plasma testosterone occurred immediately post exercise and saliva 10 minutes post exercise suggesting a similar lag time to cortisol.

The approximate 10 minute delay in peak salivary hormone measures compared to plasma does not agree with the immediate diffusion of these hormones in pharmaceutical studies. However, the pharmaceutical studies mentioned were performed during resting conditions, and during and after acute anaerobic exercise research has shown that saliva flow rate may decrease (Mackinnon *et al.,* 1993). This is suggested to be due to increased sympathetic activity during intense exercise, which causes vasoconstriction in the arterioles that supply the salivary glands, resulting in lower salivary volume (Chicharro *et al*., 1998). The reduced blood flow may explain the delay in cortisol and testosterone delivery and diffusion from blood into the saliva found after acute exercise. However, some studies have shown no effect of acute exercise on salivary flow rates (Pilardeau *et al*., 1990; Dawes, 1981). There is suggestion that a minimum intensity of >60% VO_{2max} is required for salivary secretion to be modified (Pilardeau *et al*., 1990; Bardon *et al.,* 1983); therefore, the aforementioned studies that showed no change may not have reached this threshold. There is suggestion that exercise after anaerobic threshold may affect blood flow to the salivary glands (Chicharro *et al*., 1998). There have been no studies looking at the correlation of urinary hormone levels with those in blood or saliva.

Benefits of salivary measurement

Salivary measurement has benefits over blood measures in stress research. Salivary cortisol and testosterone concentrations have been shown to be correlated with blood levels,

therefore salivary measurement present a valid representation of these hormones. Additionally, salivary measures are often indicative of the free or 'biologically active' biomarkers as they diffuse from the blood into the saliva and are not bound by albumin. Therefore, they may indicate the freely available hormones that initiate the body's stress response (Humphrey and Williamson, 2001). Finally, sample collection is less invasive therefore less likely to induce a stress response (Kraemer *et al.*, 2001) and samples can be collected by patients or study participants.

Issues with salivary measurement

A key issue with salivary measurement is contamination of samples through blood leakage due to microinjury in the oral cavity (Malamud and Tabak, 1993). Different protocols can be utlised to screen for contamination, ranging from visual inspection of discolouration, detection of haemoglobin with testing strips to testing for the presence of transferrin with immunoassay. Kivlighan *et al*., (2004) studied the above methods and their relationship with cortisol and testosterone measurement in saliva. Results revealed that cortisol levels did not change in response to minor or moderate microinjury. However, testosterone levels increased due to blood contamination and this was detected more readily with a tranferrin immunoassay rather than haemoglobin levels or discolouration. It is suggested that an additional amount of care may need to be taken to ensure that salivary testosterone immunoassay results are accurate (Schwartz and Grainger, 2004).

1.3 Analysis of stress biomarkers

1.3.1 Analytical techniques and hormones

Analytical techniques have developed over time with radioimmunoassay (RIA) the earliest developed and validated technique in the analysis of stress hormones. RIA, developed by Yalow and Berson (1959), involves making a known quantity of antigen radioactive, this is then mixed with a known amount of antibody for that antigen and the two chemically bind. Following this, a sample containing an unknown amount of antigen is then added and this competes with the radioactive labelled antigen for binding sites. Increasing amounts of non-radioactively labelled compound result in a concomitant decrease in the amount of radioactive compound that binds to the antibody. The amount of bound compound is then measured with a gamma counter. These readings can be used to create a calibration curve and subsequently concentrations of unknown samples are extrapolated from this (Hawker,

1973). RIA can be accurate and highly reliable (Furuyama *et al.,* 1970; Dufau *et al.,* 1972) with variation between and within hormone assays of < 10% if carried out stringently (Raff *et al.,* 2002; Wang *et al.,* 2004; Garcés *et al.,* 2008). Blood and saliva samples have been tested and multiple steroids measured in the same assay (Rosner *et al.,* 2007). The LOQ for RIA has been shown to be <0.15 ng/mL for testosterone (Wang *et al.,* 2004) and, 0.5 ng/mL for cortisol (Liening *et al.,* 2010). However this method can be cumbersome and time consuming and uses radioactive material (Stanczyk *et al.,* 2007); additionally, the labelled antigen may be less stable than its unlabelled counterpart and degrade in a biological matrix. Despite the low cost of measuring samples once equipment is in place (Landon and Moffat, 1976), in the late 1970s RIA gave way to enzyme-linked immunosorbent assays (ELISA) with initial analysis of immunoglobulin G (Engvall and Perlmann, 1971) and later development of an early cortisol assay (Comoglio and Celada, 1976).

ELISA are simple, convenient, and less expensive than RIA and have a high throughput when automated (Rosner *et al.,* 2007). Briefly, a plate is coated with antibodies specific to the compound of interest and an enzyme-linked compound competes with known/unknown unlinked amounts of that compound for antibody binding sites. Following this the unbound compound is washed away and bound compound is measured by its reaction with a substrate illustrated by a colour change. Absorbance values are read with a plate reader and calibration curve constructed for quantification (Salimetrics, 2011). However, ELISA often overestimate steroid concentration due to a lack of sensitivity of the antibody involved (Stanczyk *et al.,* 2007) and differences between the sample and standard solution matrix may also affect the results. Furthermore, certain steroids may not be released efficiently from sex-hormone binding globulin with the specific reagent supplied by the kit manufacturer and this could underestimate levels (Stanczyk *et al.,* 2007).

Despite the potential for overestimation, until recently ELISA was the predominant method for measurement of steroid hormones. However, since the late 1970s mass spectrometry (MS) has developed and this has now become the gold standard for detection and quantification in the pharmaceutical industry including: drug development and discovery, environmental assays and many biochemical applications including steroid analysis (Kushnir *et al.,* 2011). The basis of mass spectrometry is the production of ions (for example by electrospray ionisation, Fenn, 1990) that are subsequently separated (for

example in a quadrupole mass analyser, Yost and Enke 1978) according to their mass to charge ratio (*m/z*) (commonly in positive ion mode as the molecular mass plus one proton). Once separated the intact ions can be fragmented and the resulting ions detected (tandem MS analysis). The resulting mass spectrum is a product of the detected ions according to their *m/z* and relative abundance. The coupling of powerful separation techniques such as gas chromatography (GC) and high performance liquid chromatography (LC); commonly high performance liquid chromatography (HPLC) to mass spectrometry has resulted in the ability to accurately quantify steroid hormones in a biological matrix by selected (SRM, Thevis and Schanzer, 2007) or multiple (MRM, Chang *et al.*, 2003) reaction monitoring with stable isotope labelled analogues.

1.3.2 Mass spectrometry and steroid analysis

Recently there have been an abundance of studies attempting to detect and quantify steroid hormones such as cortisol and testosterone with mass spectrometry coupled to GC (table 1.2) or LC without derivatisation (table 1.3).

1.3.3 Gas chromatography mass spectrometry (GC-MS)

GC was originally developed by Gohlke and McLafferty in the late 1950s (Gohlke, 1959). In GC-MS analytes are separated in a heated column according to their boiling point, ionised and detected by a mass spectrometer. Analysis with GC-MS requires derivatisation in order to transform the analytes into thermally stable volatile analytes required for ionisation with silylation, the most common derivatisation method. Analytes are commonly ionised with electron ionisation (EI) and ions are bombarded with fast electrons generated from an electrically heated filament; they are accelerated through a potential difference of around 70 V and this gives electrons with 70 eV of energy. Positive ionisation is most common as electrons produced by the source collide with the sample and remove an electron, thus creating a positively charged ion with one unpaired electron (Dempster, 1921). Sufficient volatility vaporises the sample at source temperature and are steered towards the mass spectrometer. A magnet positioned across the source chamber creates a spiral in the electron beam and increases the chance of interaction between the beam and analyte gas. After entered the mass spectrometer analytes are detected according to their *m/z* ratio.

Cortisol and testosterone are thermally stable and can be derivatised to become voltatile compounds therefore they are suitable for analysis with GC-MS. Current literature contains GC-MS methods to quantify cortisol and/or testosterone in plasma and serum; however no studies have measured these hormones in saliva (table 1.2). Derivatisation methods for these hormones include combinations of N,O-Bis(trimethylsily)trifluoroacetamide (BSTFA) and Trimethychlorosilane (TMCS)*;* N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) Trimethylsilylimidazole (TMSI), and 1,4-dithioerythritol/trimethyliodosilane (DTE) as well as use of pentafluoropropanol (PFP), Pentafluoropropionic anhydride (PFPA) or heptafluorobutyroyl (HFB). Comparison of MSTFA and BSTFA has shown the MSTFA is more efficient to derviatise steroid hormones overall (Bowden *et al*., 2009).

Table 1.2. Examples of studies examining cortisol and testosterone with GC-MS

Author	**Hormone**	**Medium**	**Derivatisation**	**DL**	**QL**
Fitzgerald *et al*., (2010)	Testosterone	Serum	BSTFA/TMCS		0.05 ng/mL
Hansen *et al*., (2011)	Testosterone	Plasma and Serum	MSTFA/TMSI/ DTE		0.2 ng/mL
Legrand *et al*., (1995)	Testosterone	Plasma	PFP		0.59 ng/mL
Magnisali *et al.*, (2008)	Cortisol and testosterone	Serum	MSTFA/TMSI/ DTE	C=0.05 ng/mL, T= 0.04 ng/mL	C=0.25 ng/mL T=0.1 ng/mL
Shibasaki *et al*., (2008)	Cortisol	Plasma	HFB		1 ng/mL
Taieb *et al*., (2003)	Testosterone	Serum	HFB	0.04 ng/mL	
Yokokawa *et al.*, (2009)	Testosterone	Plasma	PFPA	1.0 ng/mL	2.0 ng/mL

Cortisol and testosterone can be derivatised for analysis with GC-MS; though no technique has quantified both analytes simultaneously in the range required for salivary measurement. Derivatisation commonly occurs by enolisation-silylation with chemical modification of ketone and hydroxyl groups, or through oximation- silylation with modification of ketone groups alone (Fang *et al*., 2010). Additionally, sample preparation

and chromatographic run times can be time consuming in this analytical method, as samples travel through the column slowly and the samples must be derivatised prior to analysis. Furthermore, recent developments of liquid chromatography coupled to mass spectrometry eliciting fast run times and less sample preparation have meant that recent quantification employs this method rather than GC-MS (Kutsukake et al., 2009; Matsui *et al*., 2009).

1.3.4 Liquid chromatography mass spectrometry (LC-MS)

Liquid chromatography separation is based on the selective distribution of analytes between a liquid mobile phase and a stationary phase. Retention time and ionisation of analytes can be affected by the chromatography chosen in the liquid phase and interaction with the stationary phase (column). LC is linked to a MS by an interface such as electrospray ionisation (ESI). The interface controls ionisation and transfer of ions to the detector in the mass spectrometer. The majority of methods for quantitative analysis use the triple quad mass spectrometer. Quantitative analysis with tandem mass spectrometry is typically performed in MRM mode with both mass analysers fixed on transmission of the compound specific precursor and product ions (Busch *et al.,* 1988). This mode offers highly selective and sensitive operation as the mass analysers transmit only ions characteristic of the target analyte and remove most of the chemical noise. A combination of chromatographic separation with LC and detection with MS/MS can achieve one of the highest analytical specificities and sensitivities available in clinical laboratories (Kushnir, 2011). Single quadrupole techniques (LC-MS) are also used for trace analysis and have the advantage of being less expensive than tandem mass spectrometry (LC-MS/MS) techniques as they do not require pure gas for collision activated dissociation (Soler *et al.*, 2005). However, analysis with LC-MS does require greater care with sample preparation and chromatography, as unlike LC/MS/MS, chemical noise is not reduced by fragmentation. Sensitivity and selectivity can be improved in LC-MS analysis with selective reaction monitoring rather than scanning across the full *m/z* range.

Table 1.3 Studies examining cortisol and testosterone with LC-MS (no derivatisation)

Author	Technique	Hormones	Medium	LOD	LOQ
Baid *et al*., (2007)	LC-MS/MS	Cortisol	Saliva	0.4 ng/mL	
Borrey *et al*., (2007)	LC-MS/MS	Testosterone	Serum		0.01 ng/mL
Cawood *et al*., (2005)	ID-LC-MS/MS	Testosterone	Serum		0.09 ng/mL
Chen *et al*., (2009)	LC-MS/MS	Testosterone	Serum		0.056 nmol/L
De Palo *et al*., (2009)	HPLC	Cortisol	Saliva		0.036 ng/mL
DiFrancesco *et al*., (2007)	LC-MS/MS	Cortisol	Plasma		3.60 ng/mL
Fanelli *et al*., (2010)	ID-LC-MS/MS	Cortisol and testosterone	Serum		C=0.24 ng/mL T=0.02 ng/mL
Fitzgerald *et al*., (2010)	LC-MS/MS	Testosterone	Serum		0.05 ng/mL
Gallagher *et al*., (2007)	LC-MS/MS	Testosterone	Serum		0.07 ng/mL
Higashi *et al*., (2005)	LC-MS/MS	Testosterone	Serum	0.02 ng/mL	
Hogg *et al*., (2005)	LC-MS	Testosterone	Saliva	0.2 ng/mL	0.5 ng/mL
Ionita *et al*., (2009)	LC-MS/MS	Cortisol	Plasma		1.0 ng/mL
Ionita *et al*., (2010)	LC-MS/MS	Cortisol	Plasma		0.1 ng/mL
Jonsson *et al*., (2003)	LC-MS/MS	Cortisol	Saliva		0.1 ng/mL
Kataoka *et al*., (2007)	LC-MS	Cortisol	Saliva	0.005 ng/mL	0.017 ng/mL
Kutsukake *et al*., (2009)	LC-MS/MS	Cortisol and testosterone	Saliva		C=0.01 ng/mL T=0.005 ng/mL

Table 1.3 cont. Studies examining cortisol and testosterone with LC-MS (no derivatisation)

Author	Technique	Hormones	Medium	LOD	LOQ
Lee *et al*., (2010)	LC-MS/MS	Cortisol	Saliva		0.2 ng/mL
Li *et al*., (2008)	LC-MS/MS	Cortisol	Saliva		0.18 ng/mL
Ma *et al*., (1997)	LC-MS	Testosterone		0.05 ng/mL	
Matsui *et al*., (2009)	LC-MS/MS	Cortisol and testosterone	Saliva		C=0.01 ng/mL T=0.005 ng/mL
Moal *et al*., (2007)	LC-MS/MS	Testosterone	Serum		0.05 ng/mL
Owen *et al*., (2010)	LC-MS/MS	Cortisol	Saliva		0.72 ng/mL
Perogamvros *et al*., (2010)	LC-MS/MS	Cortisol	Saliva		0.14 ng/mL
Shirasihi *et al*., (2008)	LC-MS/MS	Testosterone	Serum		0.02 ng/mL
Singh *et al*., (2008)	LC-MS/MS	Testosterone	Serum		0.07 ng/mL
Taylor *et al*., (2010)	LC-MS/MS	Cortisol	Urine		25 ng/mL
Wang *et al*., (2008)	LC-MS/MS	Testosterone	Serum		0.2 ng/mL
Yasuda *et al.,* (2008)	LC-MS/MS	Cortisol	Saliva	<0.03 ng/mL	

Various methods have been reported to detect and quantify cortisol and testosterone in blood and saliva (table 1.3). The majority of studies focus on measuring cortisol with different applications including: diurnal variation (De Palo *et al.,* 2009); Korean population (Lee *et al.,* 2010); obese individuals (Baid *et al.,* 2007) and the stress responses (Katoaka *et al.,* 2007). Salivary cortisol studies have reported limit of detection (LOD) between 0.017 ng/mL (Katoaka *et al.,* 2007) and 0.72 ng/mL (Owen *et al.,* 2010). The former study utilised LC-MS measurement however most studies have been with LC-MS/MS. However, the low limit of quantification (LOQ) achieved with LC-MS suggests detection without fragmentation provides sufficient sensitivity to measure salivary cortisol. Additionally, a variety of machines have been employed ranging from the Quattro Micro (Waters, UK) to API 4000 (ABSciex, UK). This variation suggests that many machines offer the required specifications to obtain sufficient sensitivity to quantify cortisol in saliva. The main difference between the mass spectrometers relates to their interface, with newer machines employing more heaters to improve solvent evaporation during electrospray and therefore increase ionisation efficiency. More ions reaching the detector lead to a proportional increase in signal strength.

Method run time is also an important parameter to consider and introduction of ultra performance liquid chromatography (UPLC) has offered new possibilities in liquid chromatography, especially concerning decreased run time and solvent consumption. A UPLC chromatographic system is designed to withstand high system back-pressures, therefore higher flow rates can be utilised. This technique employs the principle of Van Deemter equations which states that the efficiency of chromatographic process is proportional to particle size decrease. When explaining band broadening the model describes the relationship between height equivalent of theoretical plate (HETP) and linear velocity; this is partly dependent on a diameter of particle packed into the analytical column. Smaller particle diameter can significantly reduce HETP which results in higher efficiency and the flatter profile of Van Deemter curve (Van Deemter, 1956). Using UPLC, it is now possible to take full advantage of chromatographic principles to run separations using shorter columns, and/or higher flow rates for increased speed, with superior resolution and sensitivity (Patel *et al.,* 2010). Introduction of UPLC to steroid analysis has reported cortisol quantification in less than five minutes (Perogamvros *et al.,* 2010). Establishing chromatography with retention time between two to four minutes is

important to remove analyte peaks from the solvent front which can lead to improved S/N ratio, minimise ion suppression and improve assay sensitivity. No published studies have currently utilised UPLC to quantify cortisol and testosterone simultaneously in blood or saliva.

Few studies have quantified salivary testosterone; this is likely to be due to lower salivary concentrations than cortisol and lower proton affinity. Two studies have successfully quantified salivary testosterone without derivatisation (Hogg *et al.,* 2005; Yasuda *et al.,* 2008). However the work by Hogg *et al*., (2005) has limitations for use in human studies as it measures dolphin saliva and the LOQ is 0.5 ng/mL, which is above the salivary reference range for men and women. Conversely, Yasuda *et al*., (2008) presents measurement applied to healthy men and those suffering from late onset hypogonadism (LOH) or testosterone deficiency. They collected 1 mL saliva and concentrated it 10 times before analysis with an API 4000 triple quad mass spectrometer (ABSciex, UK). They reported a LOQ of <30 pg/mL which is sufficient to quantify salivary testosterone in male samples. However to improve efficiency and reduce costs of sample analysis, the growing trend is for measurement of multiple hormones from one sample. Limited studies have measured salivary cortisol and testosterone simultaneously; although, two recent studies present measurement of both hormones. Kutsukake *et al*., (2009) measured salivary cortisol and testosterone with LC-MS/MS and they revealed LOQ of 10 pg/mL for cortisol (lower reference value in humans 1000 pg/mL; Aardal and Holm, 1995) and 5 pg/mL for testosterone (lower reference value in humans 6 pg/mL; Dabbs, 1990). However, a limitation of this study was its focus on saliva obtained from chimpanzees extracted from a rope and further validation is required to transfer this method to human saliva. A second study by Matsui *et al*., (2009) addressed this issue and measured salivary cortisol and testosterone in humans using a similar method employed by Kutsuake and colleagues, reporting identical LOQ with application to LOH men. Despite one established MS method to quantify both cortisol and testosterone in human saliva, there are no studies which have applied this quantification to stress or exercise. Only one study (Katoaka *et al.,* 2007) has investigated the response of cortisol to 30 minutes running.

Additionally, given the variation in mass spectrometer specification and notably the interface employed affecting ionisation; transfer of this method to other laboratories is

limited to those possessing a machine with similar ionisation efficiency. Katoaka *et al*., (2007) reported a single quadrupole MS method was able to quantify testosterone, and given its cost effectiveness, new methods to measure cortisol and testosterone simultaneously in saliva with this method would be beneficial. Finally, approval as the gold standard in pharmaceutical and clinical settings has encouraged investigation of other potential uses of LC-MS in salivary hormone analysis.

1.3.5 Improving LC-MS sensitivity

Sample preparation

Despite the benefits of salivary measurement, like other biological fluids it can affect ionisation during electrospray through what are termed as 'matrix effects'. Matrix effects are the alternation of ionisation efficiency in the presence of co-eluting substances (Tang and Kebarle, 1993). Matrix effects can affect the development of MS methods as they may lead to ion suppression, inhibiting ionisation and sensitivity during ESI with adverse effects on LOQ (Buhrman, 1996). Ion suppression can be minimised by cleaning the sample prior to analysis with techniques such as solid phase extraction (Bonfiglio *et al.*, 1999).

Derivatisation

Derivatisation in methods for analysis of steroids could be beneficial because of the challenges involved in measuring endogenous concentrations of many of the steroids. Commonly, keto and hydroxyl groups are targeted for derivatisation (Kushnir *et al.*, 2006). Modifications to an analyte structure can change its proton affinity and facilitate improved ionisation. Derivatisation has produced an improvement in detection limit of salivary analytes of 5-10 times for cortisol (Yamashita *et al.*, 2007), four times for testosterone and 2000 times for DHEA (Shibayama *et al.*, 2008) (table 1.4).

Table 1.4 LC-MS studies measuring testosterone (with derivatisation)

Author	Technique	Medium	LOQ	LOQ
Higashi *et al.,* 2005	HMP + TFA	Serum	0.03 ng/mL	
Kushnir *et al.*, 2006	Hydroxylamine	Serum		0.01 ng/mL
Kushnir *et al.*, 2010	Hydroxylamine	Serum	0.005 ng/mL	0.01 ng/mL
Licea-Perez *et al.*, 2008	2,3, pyridine dicarboxylic anhydride	Serum		0.01 ng/mL
Shibayama *et al.*, 2008	HMP + TFA	Saliva		0.01 ng/mL
Yamashita *et al.*, 2009	DAP, MNBAn, THF	Serum		0.05 ng/mL

Testosterone has commonly been derivatised prior to LC-MS analysis to detect low levels in certain populations. Kushnir *et al.,* (2006, 2010) analysed serum testosterone on an API 4000 triple quad mass spectrometer (Applied Biosystems/Sciex) and found quantification limits of 0.01 ng/mL (10 pg/mL) after derivatisation with hydroxylamine (around the lower reference range for women in saliva). A similar quantification limit was discovered in saliva by Shibayama *et al.,* (2008) who used 2-hydrazino-1-methylpyradine (HMP) and trifluroacetic acid (TFA) to derivatise testosterone, subsequently improving sensitivity.

1.4 Hormone quantification

1.4.1 MS quantification

Bioanalytical techniques such as LC-MS can employ various techniques to quantify an unknown analyte; however, the main methods used are either standard addition on adding an internal standard. Firstly, standard addition involves spiking a series of unknown samples with known amounts of analytical standard in increasing quantities. Each spiked aliquot must be extracted and processed to give final extract solutions with a fixed final volume. The total amount of analyte is a combination of the spiked amount and unknown amount. The response for each spiked sample (y-axis) is plotted against the amount of added standard in each sample (x-axis) and a best fit line determined; the intercept should correspond to where the added amount of standard is zero. The desired quantity (unknown) is determined from the (in principle negative) intercept on the axis representing the amount of added sample (x-axis) (Figure 1.05). Despite providing the ability to account for ionisation suppression, one major limitation of this method is the large amount of sample required and time involved.

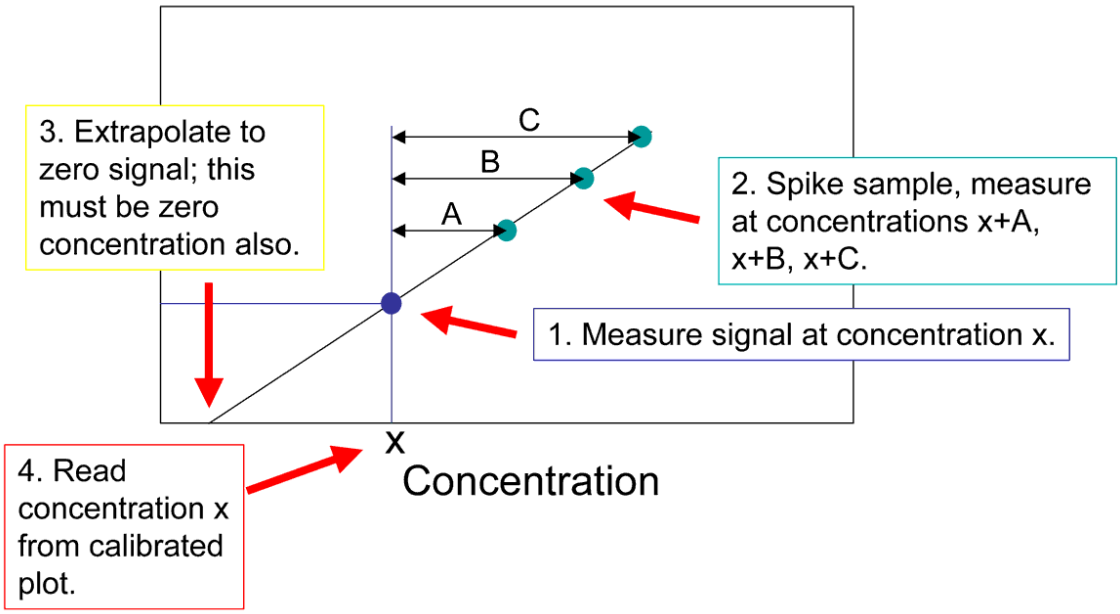


Figure 1.05 Example of standard addition method quantification method

A more widely used method in LC-MS is inclusion of labelled internal standards. This method works on the principle of adding a fixed amount of internal standard (IS) (commonly a deuterated form of the analyte being measured) to both the calibration standards and unknown samples. The responses due to the analyte standard and IS are measured in the same chromatogram run for each of the spiked calibration solutions and unknowns. The response ratio (analyte/IS) (y-axis) are plotted vs. the concentration of the calibration standards (x-axis) and the ratio of the unknown samples (analyte/IS) is used to calculate the concentration from the calibration curve regression equation (figure 1.06).

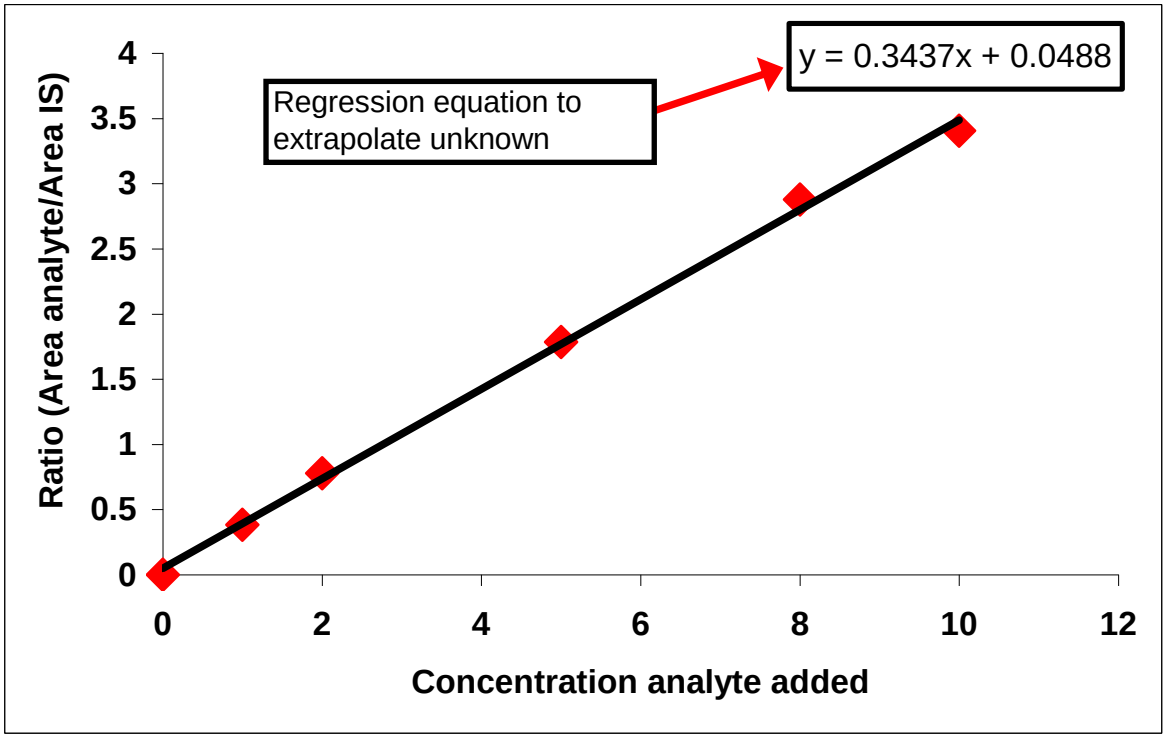


Figure 1.06 Example of peak area ratio calibration graph

It is important to choose a stable isotope labelled IS with similar physico-chemical characteristics to the analyte which will in turn offer similar elution patterns. This method has advantages including minimising/masking variations resulting from sample preparation and extractions (Wieling, 2002). Internal standard quantification is also the most reliable analytical method for high throughput trace analysis and reduces problems arising from ionisation suppression that can affect sensitivity and response of the MS (Boyd *et al.*, 2008). Given the advantages of IS over standard addition, including reduced time and sample volume, the IS method appears more efficient for quantification of cortisol and testosterone in saliva.

1.4.2 Peak measurement

Accurate quantification involves accurate peak integration. Quantification in mass spectrometry is commonly through measurement of peak area however occasionally this may be measured by peak height. Peak area is a measure of the solute quantity if the solute elutes intact and is linearly detected. Peak height is an alternative measure of solute quantity, although it is only linear over a small range but can be used to quantify peaks that are not particularly symmetrical, as long as the shapes do not change. Peak height has advantages for the measurement of small overlapping peaks. Peak area is the common

choice with a large S/N as unlike peak height as it is not susceptible to peak asymmetry and it has a greater linear range (Dyson, 1998).

Limits of detection and quantification are commonly measured using signal to noise (S/N) ratio. Noise is described as erratic variations in detector output that occur rapidly relative to the timescale of a chromatographic peak width, regardless of whether there is any analyte present. Intrinsic noise describes the electric fluctuations of an MS machine and chemical noise the 'chemical background'; the latter are small variations in chemical composition of the mixture flowing into the mass spectrometer. Peak-to-peak measurements are commonly used which encompass all contributions of background on either side of a peak (Boyd *et al.,* 1998). S/N ratio is the ratio between peak height (h_{signal}) and noise levels (h_{noise}) (figure 1.07). Accepted guidelines for detection are a S/N ratio of 3 and for quantification S/N of 5-10 (ICH, 1996).

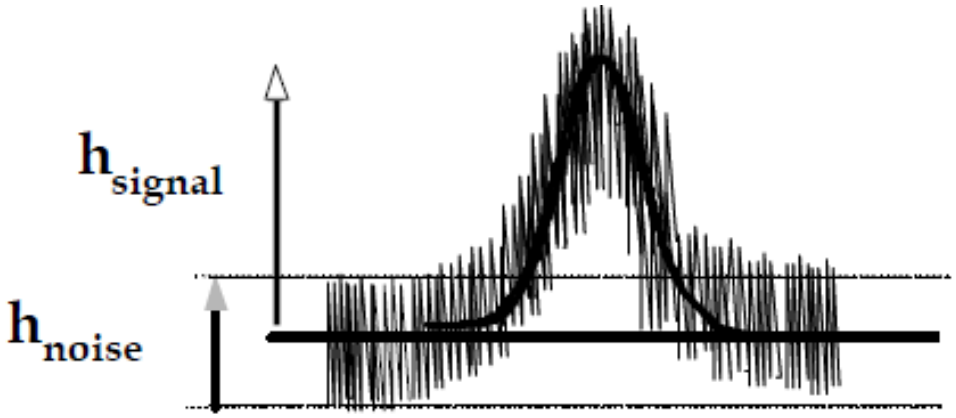


Figure 1.07 Illustration of chromatogram signal and noise (Levin, 2011)

1.4.3 Method validation

Assessing the acceptable performance of an analytical method is required before it can be put into practice. Validation experiments for mass spectrometry commonly include an evaluation of precision, linearity, accuracy, LOQ and LOD according to published guidelines (ICH, 1996; US Department of Health, 2001). Before implementing a mass-spectrometry based assay it should be compared to an available assay already in use with a range of samples.

1.5 Method comparison: MS versus ELISA

Mass spectrometry assays can produce high sensitivity, specificity and measurement of multiple analytes in a small sample volume with high throughput (Rosner *et al.,* 2007). Also, an advanced LC-MS/MS assay is capable of measuring 12 steroids in serum simultaneously within 11 minutes (Guo *et al.,* 2006) and other studies have shown profiles of numerous hormones from one samples (De Palo *et al.,* 2009; Shibaski *et al.,* 2008; Shibayama *et al.,* 2008; Wang *et al.,* 2008; Yamashita *et al.,* 2009). Dissimilarly, ELISA can only measure one analyte per assay.

Prior to 2005 immunoassays were the predominant methodology to analyse testosterone in samples from all population groups and show acceptable performance in samples from healthy men; however they have also been shown to lack sufficient sensitivity and specificity to measure low levels of testosterone in women. Taieb *et al.,* (2003) found a 46% over estimation of testosterone in women with ELISA compared to Isotope-Dilution gas chromatography–mass spectrometry. Similarly, Fitzgerald and Herold (1996) and Van Uytfanghe *et al.,* (2005) compared direct assays with GC-MS/MS and again found an overestimation and Wang *et al.,* (2004, 2008), Moal *et al.,* (2007) and Taieb *et al.,* (2003) found similar results when comparing with LC-MS/MS. Furthermore, in an accompanying editorial to Taieb *et al.,* (2003), Herold and Fitzgerald (2003) claim that guessing is as good for assessing testosterone in women given that assays can miss a target value by as much as 200-500%; this finding is also supported by Miller *et al.,* (2004). A downside of MS is the high cost of instrumentation and time to develop assays can be restrictive; however, once installed MS allows single samples to be analysed immediately without the need to fill an ELISA plate. MS is already used routinely in hospitals and to test individuals' testosterone levels during doping control (Shackleton *et al.,* 1997, Thevis *et al.,* 2005). Although this technique has been slow to enter exercise research primarily due to the high cost of machinery and time involved in method development and validation. In relation to exercise studies cortisol and testosterone have been measured in urine with GC-MS (Yap *et al.,* 1996; Pucsok *et al.,* 2005; Timon *et al.,* 2008) as well as LC-MS (Taylor *et al.,* 2010), though as yet no studies have been published in relation to exercise and salivary levels.

The studies above all use correlational analysis to examine the relationship between methods; however other techniques such as agreement analysis may give a better indication of the comparability of different methods. Correlational analysis is able to indicate if there is a relationship between sets of values, however not how closely the absolute values compare (Bland and Altman, 1986). Agreement analysis is completed by calculating the mean difference between measures (d) and the standard deviation of the difference measures (s). The following equation gives the upper and lower limits of agreement: upper/lower limits of agreement = d +/- (2 x s) (Bland and Altman, 1986, p308).

1.5.1 Application to exercise

Evidence suggests MS is the gold standard for measuring hormones in pharmaceutical, clinical and blood doping settings (Kushnir *et al*., 2011); however, the use of this valuable technique has not yet been applied to analysis of blood or salivary cortisol or testosterone in studies from sport and exercise science. As previously mentioned, physiological stress can elicit changes in hormone levels, particularly in response to exercise. Measurement of biomarkers such as cortisol and testosterone can aid understanding of the hormonal exercise response to training and multiple studies have examined this response within different sporting contexts.

1.6 Cortisol and testosterone responses to acute exercise

1.6.1 Cortisol and acute exercise

It is well established that cortisol increases after exercise, with the majority of studies reporting this (Table 1.5). The main function of cortisol is to maintain blood glucose levels during physical exercise by acting on skeletal muscles and adipose tissue to increase amino acid and lipid mobilisation (Galbo, 2001; Wolf, 2001). Cortisol also stimulates the liver to produce enzymes involved in gluconeogenesis allowing conversion of amino acids and glycerol to glucose and glycogen. However, some studies showed a decrease in cortisol after exercise (Meeusen *et al.*, 2004; Beaven *et al.*, 2008). These results may have been affected by diurnal variation as the samples were collected in the morning but exercise did not commence immediately; therefore, the decrease may have represented the natural sharp decline in cortisol throughout the morning. Similarly, an anticipatory increase in cortisol may have produced a false decline which could not be eliminated due

to lack of baseline average values. There are various potential reasons for no change in cortisol, including low exercise intensity, short duration, the exercise mode and participants training status. The intensity and duration of exercise appear to be important factors impacting the cortisol response, as well as exercise type and muscle mass involvement.

1.6.2 Cortisol and exercise intensity

The overall consensus supports the hypothesis of a threshold of exercise intensity above which cortisol levels increase. Jacks *et al.,* (2002) studied the response of salivary cortisol to one 60 minute cycle ergometry bouts at 45%, 62% and 76% VO_{2max} and found a significant increase after 59 minutes of exercise at 76% VO_{2max} only. This outcome is supported in a shorter duration exercise study (Allgrove *et al.,* 2008) examining cycling trials (average 22.3 minutes) at 50% and 75% VO_{2max}; the latter trial showed a significantly higher increase in salivary cortisol levels one hour post exercise when compared to the other trials. In a group of healthy schoolchildren, Budde *et al.,* (2010) also found those who ran for 12 minutes at 70-85% maximum heart rate showed an increase in salivary cortisol levels, whereas at 50-65% there was no increase. Further research (Hill *et al.,* 2008) examined 12 active men performing 30 minutes of cycle ergometry at 40, 60 and 80% VO_{2max}; only the moderate (60%) and high (80%) intensity trials elicited a significant increase in serum cortisol levels. Support by studies from O'Connor and Corrigan (1987) and Rudolph and McAuley (1998), with increases in serum and salivary cortisol levels after 30 minutes cycle ergometry at 75%VO_{2max} and 30 minutes running at 60% VO_{2max} respectively, suggest that in a highly controlled study there is a threshold of around 60% VO_{2max} to elicit an increase in salivary cortisol levels over 20 minutes or more of continuous cycling or running. However, there is evidence that this threshold may not be conclusive (Jacks *et al.,* 2002; Budde *et al.,* 2010).

The variation of findings in studies considering the cortisol response and exercise intensity may be due to the use of percentage VO_{2max} as controlling variable. In 1928, Hill claimed that expressing a given aerobic energy yield in percentage VO_{2max} was a way to normalise people with different exercise capacities. This is supported by Costill *et al*., (1973) who found that elite marathon runners exercised at a similar VO_{2max} (80-90%) to those finishing further down the field. However, there are other variations that should be considered when

exercising at a normalised percentage VO_{2max}, for example lactate threshold. Coyle *et al*., (1988) conducted a study in cyclists with a VO_{2max} of 67 ml/kg/minute and found that within the group there were participants termed high lactate responders who produced higher levels of blood lactate when exercising at 88% VO2max compared to others in the group. This characteristic resulted in the endurance of the high lactate responders being half that of the low lactate responders. The latter finding suggests that normalising to percentage VO_{2max} during a hormone study may not encompass differences in lactate threshold or training adaptations in participants. Recent research by Lansley *et al*., (2011) has investigated an alternative to percentage VO_{2max} for normalisation of exercise intensity. Their study investigated the use of gas exchange threshold (GET) which is synomomous with lactate threshold. Individials vary in GET from 40-60% maximum in untrained up to 80% in endurance athletes (Jones and Carter, 2000). Lansley and colleagues employed %Δ delta GET which is the GET plus percentage interval between the GET and VO2max. Exercise at a set % Δ resulted in significantly decreased subject variability in gas exchange, blood lactate accumulation, heart rare and RPE. Therefore, this concept could provide more accurate characterisation of exercise intensity in experimental studies designed to measure the impact of a given intervention. Additionally, if participants may begin exercising at a set percentage VO_{2max} but by the end of the trial are likely to be working at a higher proportion of their maximal oxygen uptake. This raises doubt over the true threshold intensity of cortisol release. Finally, standardising exercise intensity lacks validity when considering the hormonal response in a competition setting. It is also important to control of all factors that may influence cortisol levels including time of day, timing of sample (to encompass lag time), circadian rhythm, diet and previous exercise.

Table 1.5 Studies investigating the cortisol response to exercise

Author	Participants	Exercise	Cortisol
Allgrove *et al.,* (2008)	10 men	Cycle ergometry	↑ after 75% VO_{2max} and exhaustion trials
Beaven *et al.,* (2008)	23 rugby players	Resistance exercise	Salivary ↓ significantly
Brownlee *et al.,* (2006)	50 active men	Circuits, weights, running rowing and cycling	Blood ↑ post exercise
Budde *et al.,* (2010)	60 students	Running for 12 mins	Salivary ↑after 70-85% VO_{2max}
Cadore *et al.,* (2008)	21 middle aged men	Strength training	↑ serum in untrained
Cadore *et al.,* (2009)	10 young and 10 elderly men	Water training protocols	No change
Cordova *et al.,* (2009)	12 volleyball players	Cycling to fatigue	↑ serum levels
De Corral *et al.,* (1994)	10 male children	Cycle ergometer	Serum ↑
Daly *et al.,* (2005)	22 male athletes	Running to fatigue	Serum ↑ post exercise
Di Luigi *et al.,* (2006)	110 male athletes	90 mins various training	Salivary ↑ post training
Dimitriou *et al.,* (2002)	14 male swimmers	Swimming intermittent	↑ salivary levels
Eliakim *et al.,* (2009)	14 male and 14 female volleyballers	One hour volleyball	No change
Eloumi *et al.,* (2003)	20 male rugby players	Competitive match	↑ sharply during comp
Farzanaki *et al.,* (2008)	11 female gymnasts	One or two training sessions	Salivary ↑ after 2 sessions
Franca *et al.,* (2006)	20 male athletes	Marathon race	↑ post race

Table 1.5 cont. Studies investigating the cortisol response to exercise

Author	Participants	Exercise	Cortisol
Fry and Lohnes (2010)	4 trained men	Weight lifting session	No change
Hackney and Viru (1999)	17 men	Running or cycling	↑ after moderate and high intensity
Hill *et al.,* (2008)	12 men	Cycle ergometry	↑ after 60% and 80% VO_{2max}
Hough *et al.,* (2011)	10 men	Cycling and resistance	↑ in 50/80 and fatigue trials
Isprilidis *et al.,* (2008)	24 male footballers	Football match	↑ post match
Jacks *et al.,* (2002)	10 active men	Cycle ergometry	↑ after 76% VO_{2max}
Karkoulias *et al.,* (2008)	11 marathon runners	Marathon race	Serum ↑ post race
Kokalas *et al.,* (2004)	Six male rowers	Various rowing sessions	↑ post endurance protocol
Kraemer *et al*., (1993)	Eight men	Resistance exercise	↑ after high total work trial
Kraemer *et al*., (1999)	7 powerlifters, 12 untrained men	Resistance exercise	↑ untrained
Le Panse *et al.,* (2010)	26 elite power lifters	Power lifting competition	↑ after bench press
Marinelli *et al.,* (1994)	Six athletes	Marathon race	↑ post race
McMillan *et al*., (1993)	21 trained and untrained men	Resistance exercise	↑ untrained
Meeusen *et al.,* (2004)	Seven cyclists	Two max exercise tests pre and post training camp	Pre camp ↓ during test 1 and ↑ during 2^{nd}. Post camp ↓ in both tests
Moreira *et al.,* (2009)	22 male footballers	Football match	No change

Table 1.5 cont. Studies investigating the cortisol response to exercise

Author	Participants	Exercise	Cortisol
Moreira *et al.,* (2010)	17 male athletes	Kickboxing match	↑ post match
O'Connor and Corrigan (1987)	Eight men	Cycle ergometry	↑ serum and salivary levels post exercise
Passelergue and Lac (1999)	15 young wrestlers	Two day wrestling competition	↑ before and after the comp
Petraglia *et al.,* (1988)	27 male athletes	Athletics meeting	↑ after 10km, 1500m and 100m
Rowbottom *et al.,* (1995)	18 male triathletes	Run to exhaustion and cycle ergometry	Serum ↑ post exercise
Rudolph and McAuley (1998)	13 male cross country runners	30 mins running	↑ post exercise
Sari-Sarraf *et al.,* (2007)	10 males	Football training	Post exercise ↑ not sig
Stuempfle *et al.,* (2010)	44 male athletes	Ultra endurance race	↑ post race
Thomas *et al.,* (2009)	17 schoolchildren	Intermittent cycling	Salivary ↑ post exercise
Thomas *et al.,* (2010)	19 girls	Intermittent cycling	No change
Tremblay *et al.,* (2005)	Eight men	Various running protocols	↑ post run
Viru *et al.,* (1992)	82 men	Two hr cycle ergometry	↑ post exercise
Viru *et al.,* (2001)	12 endurance athletes	Cycling and running	↑ after first test
Vuorimaa *et al.,* (2008)	20 trained runners	Running protocols	↑ after tempo run in middle distance (MD) runners

1.6.3 Cortisol and exercise duration

Previous research suggests that intense exercise (>60% VO_{2max}) of >20 mins increases cortisol levels; however, during lower intensity exercise there appears to be a threshold of time to elicit an increase. Tremblay *et al.,* (2005) conducted a study into plasma cortisol response after treadmill runs of 40 minutes, 80 minutes and 120 minutes at 55% VO_{2max} in eight endurance trained males. Cortisol levels increased in response to the 120-min run in the second hour of running (22%) and showed a decline across time in the other shorter sessions. It is suggested that when exercise is performed under 60% VO_{2max}, hormonal responses will only occur when a certain amount of work is done, i.e. running for an adequate time (Viru *et al.,* 1992). Tremblay *et al.,* (2005) demonstrated this to be 80 minutes of running at low intensity (55% VO_{2max}). Research into marathon running supports the claim that long duration exercise will increase circulating cortisol levels. Franca *et al.,* (2006), Marinelli *et al.,* (1994) and Karkoulias *et al.,* (2008) all studied marathon races (various durations from three to eight hours) and found a significant increase in cortisol levels post race. This is supported by Stuempfle *et al.,* (2010) who found an increase in cortisol levels after a 161 km ultra endurance race (28.3 ± 7.4 hours). A study by Petraglia *et al.,* (1988) measured cortisol before and after an athletic competition in 10,000m, 1500m and 100m athletes where, despite a pre exercise anticipatory increase, plasma cortisol levels still significantly rose in all the athletes, with a higher increase in the 10,000m (n=8) and 1500m (n=7) compared to the 100m (n=7). These results suggest that longer duration exercise (>20 minutes) will lead to a higher increase in cortisol concentration; however, short duration exercise (>15 seconds) may still stimulate the HPA axis and cortisol release.

There is evidence that glycogen depletion is greater with an increase of intensity (Vollestad and Blom, 1985) and duration (Coyle *et al*., 1986) of exercise. There is evidence to suggest that cortisol enhances lipolysis and protein catabolism to mobilise fuels (Nindl *et al*., 2001), therefore cortisol, may provide a glycogen sparing role during exercise. A study by Grego *et al*., (2004) showed that during 80 minutes of cycling at 66% VO2max cortisol levels increased significantly after 144 minutes and this corresponded to an increase in glycerol and free fatty acid in the blood and a decrease in blood glucose. This is supported by Steensberg *et al*., (2002) who reported an increase in plasma free fatty acids and cortisol concentrations between 90 and 120 minutes of exercise in a glycogen depleted state. These studies suggest that the level of glycogen depletion may mediate the hormonal

response to exercise rather than the intensity or duration *per se*. The latter study suggests that with maximal exertion, exercise duration <20 minutes can elicit a significant cortisol response; however further investigation is required to support these findings.

1.6.4 Cortisol and intermittent exercise

The effects of intermittent exercise on cortisol levels are less clear; although, generally this exercise mode elicits an increase in cortisol levels after intermittent exercise such as swimming and running interval sessions (Dimitriou *et al.*, 2002; Vuorimaa *et al.*, 2008) and team sports such as rugby (Elloumi *et al.*, 2003). However, Eliakim *et al.*, (2009) studied the response of one hour volleyball practice on serum cortisol and found no change. Similarly, Thomas *et al.*, (2009) revealed no significant increase in salivary cortisol levels in schoolgirls who performed 6x8 second cycle sprints with 30 seconds recovery, although the same trial in boys produced a significant increase post exercise, perhaps suggesting the boys exerted themselves more during the sprints. Similarly, Moreira *et al.*, (2009) also revealed no significant change in salivary cortisol level after a competitive football match. The latter might relate to exercise intensity as a trained athlete may have a higher threshold for cortisol release in response to exercise. This is supported by Sari-Sarraf *et al.*, (2007) who studied a football training session and also found no significant increase in salivary cortisol levels. It is possible athletes may become accustomed to performing a certain type and intensity of exercise and therefore adapt (Vuorimaa *et al.*, 2008), requiring extra stress to elicit a hormone response. There are various reasons this may occur, firstly the athletes may have been overtraining and suffering from a decreased adrenal sensitivity to ACTH (Lehmann *et al*., 1997). Alternatively, after endurance training there is an increased use of fat as a fuel during exercise (Hollosky and Coyle, 1984). This is not due to an increase in free fatty acid (FFA) availability as plasma FFA mobilisation does not increase after training (Horrowitz and Klein, 2000). An increased oxidation of intra muscular triglycerides (IMTG) has been postulated as most studies have found that athletes do not oxidise more circulating FFA during exercise than their untrained counterparts (Horrowitz and Klein, 2000; Martin *et al*., 1993). Therefore, the requirement of extra exercise stress to elicit a cortisol response may be linked to more efficient utilisation of fat as a fuel at lower intensities and reduced reliance on glycogen stores and blood glucose.

Furthermore, the lack of increase may also have been due to inadequate exercise stimulus, supported by Passelergue and Lac (1999) who concluded that the observed increase after a wrestling match was a reflection of the pre-exercise anticipatory response due to the low exercise intensity. Nevertheless, many studies have shown an increase in cortisol levels. Dimitriou *et al.,* (2002) found a swimming interval session contributed to a 76% increase in salivary cortisol concentration and research by Di Luigi *et al.,* (2006) found intermittent moderate intensity football training produced an increase in salivary cortisol of 12.4% post training. In other team sport studies both Elloumi *et al.,* (2003) and Ispirlidis *et al.,* (2008) showed an increase in cortisol levels after a rugby match and football match respectively. Individually, Moreira *et al.,* (2010) studied the effect a kickboxing competition on salivary cortisol concentration and found an increase post match.

A recent laboratory study by Hough *et al.,* (2011) measured salivary and plasma cortisol and testosterone levels in four trials including two cycle ergometer interval sessions. The interval session consisted of a 55/80 trial (30 minutes alternating one minute at 55% maximum work rate and four minutes at 80% maximum work rate) and a 60/90 trial (30 minutes alternating one minute at 90% maximum work rate and one minute at 60% maximum work rate). Plasma cortisol levels increased after all trials and salivary cortisol increased after the 55/80 trial. This suggests that even in an intermittent exercise session work > 60% maximum must exceed 20 minutes duration to elicit a cortisol response. In their other interval session there was no increase in salivary cortisol level and the authors concluded this was due to inclusion of < 15 minutes exercise above 60% maximum. Other researchers (Vuimoraa *et al.,* 2008) investigated the effect of a tempo run (TR) or intense interval training (IT) on serum hormone levels. Serum cortisol levels increased after both trials (17% after 20 minutes of IT) and 30% and 25% for IT and TR respectively post exercise. After 10 minutes recovery the IT had increased 39% and TR 51%; suggesting the exercise induced increase in cortisol tends to take place earlier if the exercise is more intense. Overall it appears that intermittent exercise may elicit increases in blood and salivary cortisol concentrations; however, during the session an intensity of >60% VO_{2max} is required for at least 20 minutes duration.

1.6.5 Cortisol and resistance exercise

There is no clear trend regarding the cortisol response to resistance exercise. Studies show a difference in the intensity and number of repetitions employed, and the cortisol response

has been investigated by changing these parameters. After testing four different exercise protocols (4 sets of 10 repetitions at 70% 1 repetition max (1RM); 3 sets of 5 reps at 85% 1RM, 5 sets of 15 reps at 55% 1RM and 3 sets of 5 reps at 40% 1RM), Beaven *et al.,* (2008) revealed a decline in cortisol concentration across all protocols, this is comparable to Smilios *et al.,* (2003). Other researchers such as Fry and Lohnes (2010) have shown no change in cortisol levels. Their protocol involved 10 speed squats at 70% 1RM plus body weight, and this is supported by findings from Hakkinen *et al.,* (1987).

However, Cadore *et al.,* (2008) found that serum and salivary cortisol levels were both significantly increased after a resistance training bout consisting of two exercises at 75% 1RM (8 repetitions each) and two exercise at 75% and 65% 1 RM (8 and 12 repetitions respectively). Similarly, Hough *et al.,* (2011) conducted a resistance test in 10 males consisting of 8 sets of a 10 repetition max squat test. Plasma cortisol levels increased from pre-exercise to 10 minutes post exercise, this is supported by McCaulley *et al.,* (2009). In a shorter bout of competitive resistance exercise, Le Panse *et al.,* (2010) revealed a significant increase in salivary cortisol levels after three bench press attempts in an international power lifting competition ($p<0.01$), despite an anticipatory stress response evident in the women sampled. There is some consensus on the effect of training status on cortisol response; with untrained participants eliciting a higher cortisol response (McMillan *et al.,* 1993; Kraemer *et al.,* 1999). Hypertrophy protocols also appear to induce a higher cortisol response than neural lifting schemes (Kraemer *et al.,* 1993). Hypertrophy and neural (strength) protocols were descrived by Kraemer *et al*., (1990; 1993). The strength protocol was characterised by lifting a higher resistance fewer times (5RM), leading to lower volume of training overall. The hypertrophy programme consisted of a lighter resistance (10RM) but training volume was higher. The latter findings suggest that overall volume of training could be more important in relation to the acute cortisol response than intensity alone. This is supported by Nunes *et al*., (2011) who studies three strength training protocols in elite female basketball players on a continuum ranging from an endurance scheme (4 sets of 12 reps, 60% 1RM) to a strength-hypertrophy scheme (1 set of 5RM, 1 set of 4RM, 1 set of 3RM, 1 set of 2RM, and 1set of 1RM, followed by 3 sets of 10RM) and a power scheme (3 sets of 10 reps, 50% 1RM load power). They discovered that the strength-hypertrophy scheme elicited the highest increase in salivary cortisol levels, and postulate this was due to the higher overall training volume of this session leading to a higher metabolic demand. Further studies are required to establish an overall

trend for the cortisol response to resistance exercise; however, there is evidence that the cortisol response may be able to be used to evaluate the metabolic demands of different resistance exercise schemes or as a tool for monitoring training strain (Nunes *et al*., 2011).

1.6.6 Testosterone and exercise intensity

Similarly to cortisol, exercise intensity appears to affect the testosterone response to acute exercise (table 1.6). Budde *et al.,* (2010) revealed a significant increase in salivary testosterone after 12 minutes of running at 70-75% maximum heart rate (HR) but no change at a lower intensity (65%). This is supported by Kokalas *et al.,* (2004) who found a 60% increase in serum testosterone after 60 minutes rowing at blood lactate levels of 3-4 mmol.l. However, a short duration trial (10 minutes at 70% VO_{2max}) failed to elicit a change in testosterone (Viru *et al.,* 2001). These results support the hypothesis that there is a threshold of exercise intensity above which testosterone levels increase; however, the bounds of this threshold are yet to be established. The increase in testosterone could be due to increased release from the adrenal cortex or changes in vascular shift (Daly *et al.,* 2005).

1.6.7 Testosterone and exercise duration

Tremblay *et al.,* (2005) presented a 20% increase in free and total testosterone after one hour of 80 min and 120 minute runs at 55% VO_{2max}. However, after an hour of exercise there was a subsequent decline in testosterone that continued throughout recovery. The authors also revealed that testosterone levels were greater in an 80 minute run compared to 40 minute, suggesting testosterone may have a dose response relationship with exercise duration. A decrease in testosterone levels have been consistently observed following marathons (Marinelli *et al.,* 1994; Franca *et al.,* 2006; Karkoulias *et al.,* 2008) and in a study by Brownlee *et al.,* (2006). The high physiological stress of a marathon may contribute to this decrease, and testosterone levels may be related to cortisol release; therefore, a sufficient rise in cortisol levels may lead to a subsequent decline in testosterone (Brownlee *et al.,* 2005). Galbo *et al.,* (1977) suggested the decline in testosterone could be explained by a decrease in testicular blood flow as exercise is prolonged. Pharmaceutical studies manipulating levels of cortisol by venous infusion have resulted in a reduction in circulating testosterone (Bambino and Hsueh, 1981; Cumming *et al.,* 1983). This is suggested to be caused by direct steroidogenesis inhibition at the Leydig cells of the testes and/or central and peripheral disruption of the hypothalamic pituitary

gonadal regulatory axis (Hackney and Dobridge, 2003). Variations in response might also correspond to whether free or total testosterone was measured. Daly *et al.*, (2005) found that total testosterone decreased after a run to fatigue at 100% ventilatory threshold; however free testosterone increased. They suggested this could be a result of free testosterone secretion from the adrenal gland (Wilson, 1998) with a concomitant release of cortisol and testosterone from this gland. Alternatively, the relationship could be due to plasma volume shifts affecting free testosterone levels. Their results support this theory as controlling for vascular fluid shift meant free testosterone did not significantly change.

Table 1.6 Studies investigating the testosterone response to exercise

Author	Participants	Exercise	Testosterone
Ahtiainen *et al* (2005)	13 strength-trained men	Resistance exercise	No influence of rest times
Beaven *et al.,* (2008)	23 rugby players	Resistance exercise	No overall change
Brownlee *et al.,* (2006)	50 active men	Circuits, weights, running rowing and cycling	Blood ↑ post exercise
Budde *et al.,* (2010)	60 students	Running for 12 minutes	Salivary ↑after 70-85% VO_{2max}
Cadore *et al.,* (2008)	21 middle aged men	Strength training	↑total and free in untrained and free in trained
Cadore *et al.,* (2009)	10 young and 10 elderly men	Water training protocols	Salivary ↑ after intermittent exercise
Daly *et al.,* (2005)	22 male athletes	Running to fatigue	Serum ↑
Di Luigi *et al.,* (2006)	110 male athletes	90 mins various training	Salivary ↑
Eliakim *et al.,* (2009)	14 male and female volleyballers	One hr volleyball	↑ serum levels
Franca *et al.,* (2006)	20 male athletes	Marathon race	↓ post race
Fry and Lohnes (2010)	Four trained men	Weight lifting session	No change
Hough *et al.,* (2011)	10 men	Cycling and resistance	↑ in all trials bar 50/80
Isprilidis *et al.,* (2008)	24 male footballers	Football match	No change
Karkoulias *et al.,* (2008)	11 marathon runners	Marathon race	Free and total serum ↓ post race

Table 1.6 cont. Studies investigating the testosterone response to exercise

Author	Participants	Exercise	Testosterone
Kokalas *et al.,* (2004)	Six male rowers	Various rowing sessions	↑ more after endurance
Kraemer *et al.,* (1990)	Nine men	Resistance exercise	No change
Kraemer *et al.,* (1991)	Eight men and eight women	Resistance exercise	Men, serum↑
Kraemer *et al.,* (1999)	Seven powerlifters and 12 untrained men	Resistance exercise	↑ powerlifters
Le Panse *et al.,* (2010)	26 elite powerlifters	Power lifting competition	No change
Linnamo *et al.,* (2005)	Eight men and eight women	Resistance exercise	Serum ↑during heavy resistance
Marinelli *et al.,* (1994)	Six athletes	Marathon race	↓ post race
Passelergue and Lac (1999)	15 young wrestlers	Two day wrestling competition	↑ post competition
Thomas *et al.,* (2009)	17 schoolchildren	Intermittent cycling	Salivary ↑ post exercise
Thomas *et al.,* (2010)	19 girls	Intermittent cycling	No change
Tremblay *et al.,* (2005)	Eight men	Various running protocols	↑ post run
Viru *et al.,* (2001)	12 endurance athletes	Cycling and running	↑ post 2 hour run
Vuorimaa *et al.,* (2008)	20 trained runners	Running protocols	↑ after intermittent run in MD runners
Yarrow *et al.*, (2007)	22 untrained men	Resistance exercise	No change

1.6.8 Testosterone and intermittent exercise

Overall, testosterone appears to increase after intermittent exercise. Eliakim *et al.,* (2009) observed an increase in serum testosterone after a one hour volleyball training session as did Di Luigi *et al.,* (2006) after 90 minutes football training. However, Ispirildis *et al.,* (2008) found no increase in testosterone after a football match, nor did Thomas *et al.,* (2010) after a 6x8 second sprints with 30 seconds recovery on a cycle ergometer in adolescent girls. However, as stated for cortisol their identical study in adolescent boys (Thomas *et al.,* 2009) reported a significant increase in testosterone. The authors concluded that this may have been due to the very low levels of testosterone in girls and an inability to gain sufficient detection when analysing the samples. Additionally, they failed to control for the potential effect of oral contraceptives which may limit short term changes in testosterone with exercise (Enea *et al.,* 2009). A study (described previously) by Hough *et al.,* (2011), found an increase in salivary testosterone for all exercise trials including two interval sessions. Similarly, Vuimoraa *et al.,* (2008) reported that testosterone increased similarly after IT and TR in competitive runners. However, the IT only showed a significant increase in middle distance runners, (not marathon runners). The authors postulated that the post IT increase may be due to the influence of circulating catecholamines or SNS (Jezova & Vigas, 1981). In their study testosterone levels were correlated with blood lactate response after a VO_{2max} test and they hypothesised that differences in the structure of fast and slow muscle fibres regarding the affinity (Bricout *et al.,* 1999) and number (Monks *et al.,* 2006) of androgen receptors as well as testosterone-induced changes in lactate transport (Enoki *et al.,* 2006) may be responsible for the increase in testosterone. This result is supported by Kokalas *et al.,* (2004) who studied rowers and found an interval session of 4x5 minutes at 4-6 mmol.l produced a 42% increase in serum testosterone.

1.6.9 Testosterone and resistance exercise

The resistance exercise protocol design, including intensity and volume of training, appears to underpin the testosterone response (Crewther *et al.,* 2006). It has been established that schemes designed to induce hypertrophy (less resistance more sets/repetitions) result in a larger increase in testosterone than those designed to elicit neural adaptations (Kraemer *et al.,* 1991). However, this increase is not seen in women (Kraemer *et al.,* 1991). Studies that observed an increase in testosterone suggested this

may be related to the magnitude of stress of the session. In their recent review, Vingren *et al.,* (2010) proposed there is a threshold of intensity to elicit a testosterone response to resistance training (supported by studies from Yarrow *et al.,* 2007; Kraemer *et al.,* 1990; Raastad *et al.,* 2000; Hakkinen *et al.,* 1993, Linnamo *et al.,* 2005). Training volume (total workload performed) also appears to be important with a higher volume eliciting a greater metabolic demand and in turn teststoerone response (Similios *et al*., 2003). The period of rest between sessions has also been considered, as it is postulated a shorter recovery could increase metabolic demand (Kraemer *et al.,* 1990); however, this has not been found to translate into a greater increase in testosterone (Ahtiainen *et al.,* 2005). Physiologically, testosterone has anabolic properties and has been linked to muscle growth; it is thought that testosterone has direct interaction with a cytoplasmic receptor on the muscle cell which in turn initates activation of RNA synthesis, and subsequent accumulation of muscle proteins (Florini, 1985). Another important factor affecting the testosterone response to resistance exercise appears to be training status; testosterone response in strength trained individuals appears to be greater than for untrained (Kraemer *et al.,* 1999; Ahtiainen *et al.,* 2004). This suggests that perhaps trained individuals are better adapted to increase muscle growth than their untrained counterparts. However, it has also been proposed that the increase in testosterone could be a result of decreased muscle utilisation (Kraemer *et al*., 1990) therefore trained individuals may require less testosterone utilisation by the muscle to aid growth and repair. Some studies did not find a significant increase in testosterone such as Beaven *et al.,* (2008) who tested four different resistance exercise protocols (previously described); Kokolas *et al.,* (2004) who reported an insignificant serum testosterone increase of 24% after a bout of resistance training and Le Panse *et al.,* (2010) who showed no change after an international weight lifting competition. These results suggest the intensity of the exercise may not have been high or sustained enough to elicit a change. The overall consensus is that the adaptive response to strength training is likely to be multi faceted with several acute training factors (one of them hormonal) rather than a single factor. With regards testosterone, intensity and volume of training appear to be the most important factors to consider when planning a training session and additional factors such as age, and nutritional status may also be of importance (Crewther *et al.,* 2006).

1.7 Testosterone/cortisol ratio (T/C ratio)

The T/C ratio is increasingly being identified as a marker of training stress as the focus on research into metabolic aspects of fatigue increases (Petibois *et al.*, 2002). This review focuses mainly on the acute effects however the chronic effects may indicate an athlete's training status. Most studies have reported a decrease in the T/C ratio after acute exercise. Franca *et al.,* (2006) and Marinelli *et al.,* (1994) both observed a decrease after a marathon race; Elloumi *et al.,* (2003) after a rugby match; Le Panse *et al.,* (2010) after a power lifting competition and Passelergue and Lac (1999) after a wrestling competition. The latter suggest that competition and/or strenuous exercise results in a low T/C ratio which is considered as a catabolic phase i.e. breakdown of tissues. Conversely, the recovery phase (associated with a feeling of tiredness and incapacity to train strenuously) corresponds to a high T/C ratio and anabolic or muscle building phase. Passelergue and Lac (1999) discovered a high T/C ratio during recovery, which may indicate restoration of the body after the heavy exercise. One study (Di Luigi *et al.,* 2006) that found an increase in T/C ratio in adolescents playing football, proposed that the increase may have been due to the lower cortisol response in the adolescents, which could be explained by the contribution of different endocrine pathways and testes volume during the stages of pubertal development (Weise *et al.,* 2002). Overall, the ratio tends to decrease in response to an increase in cortisol levels after exercise, despite an increase in testosterone of lower magnitude than cortisol. Longer duration exercise (such as marathon running) has been shown to decrease testosterone (possibly due to a large increase in cortisol levels) which in turn leads to a decrease in T/C ratio (Franca *et al.,* 2006; Marinelli *et al.,* 1994). However, with longer duration exercise the intensity must also be considered. Repetitive strenuous acute bouts of exercise without adequate recovery may have wider relevance, and can lead to long term fatigue and reductions in performance, sometimes diagnosed as overtraining syndrome or underperformance syndrome.

1.8 Overtraining syndrome

As previously mentioned, overtraining syndrome is characterised by fatigue and underperformance precipitated by the stress of training (Budgett, 1998). T/C ratio tends to decrease in response to training or a period of intensified training and is increasingly being identified as a potential marker for diagnosis of overtraining syndrome (Banfi *et al.,* 1993). Other stresses, depression and an increased susceptibility to infections may

also be important (Budgett, 1990). Originally, Adlercruetz *et al.,* (1986) proposed that a decrease in T/C of 30% or more below resting levels may be indicative that an athlete is suffering from overtraining syndrome. However, Banfi *et al.,* (1993) tested the usefulness of the T/C ratio in elite skaters and concluded that the previous threshold for overtraining syndrome should be modified to below 18% or more. Most studies have observed a decrease in the ratio after heavy exercise or intensified training. Gonzalez-Bono *et al.,* (2002) identified that intensified training in basketball players led to a decrease; this is supported by Maestu *et al.,* (2005) in rowers, Coutts *et al.,* (2007) in rugby players and Ishigaki *et al.,* (2005) in endurance runners. Argus *et al.,* (2009) examined the response of cortisol and testosterone to rugby preseason training and a competitive season. They observed a decrease in T/C ratio across the season, and concluded the concomitant increase in testosterone may have been evidence that the endocrine system was recovering after the intense preseason training. Cortisol levels increased more than testosterone across the season and could reflect the players' lack of recovery from the previous match. Furthermore, Elloumi *et al.,* (2003) suggest five days rest or light training is required to adequately recover from the demands of a rugby match. Most studies conclude that the ratio decrease indicates an imbalance between anabolism and catabolism and is useful for monitoring the adaptation to training and ultimately progression towards overtraining. However, Urhausen and Kindermann (2002) employ that it may be more a physiological indicator of training status than an indicator of overtraining itself. This explanation is supported by studies that have observed an increase in T/C ratio (Santtila *et al.,* 2009; Tanskanen *et al.,* 2011). Tanskanen *et al.,* (2011) measured the ratio in adult army recruits and found an increase after army training (weeks 4-7); although in those recruits identified with overtraining syndrome the ratio tended to decrease suggesting that some recruits coped with the training better than others. In a similar study with army recruits Santtila *et al.,* (2009) also found an increase in the ratio. Coutts *et al.,* (2007) hypothesised that an elevated T/C ratio may indicate that an athlete is tolerating their training load because while exercise may elicit a decrease in the ratio immediately, it would then increase to indicate a rise in testosterone and anabolism i.e. during muscle repair and regeneration.

Further research is still required to clarify guidelines for the use of the T/C ratio in monitoring training and diagnosing overtraining syndrome. Recently research has been undertaken into repeated bouts of exercise for the diagnosis of overtraining syndrome.

In a study by Meeusen *et al.,* (2004), cyclists performing two graded incremental exercise tests to exhaustion separated by 4 h before (not over-reached) and after (overreached) a 10 day training camp. There was a decrease in cortisol after the first trial and no change in cortisol concentration after the second trial in overreached athletes; in addition their resting levels were higher than non-overreached athletes who demonstrated no change after the first trial and an increase after the second. Blunting of cortisol may be linked to central disruption at the hypothalamic pituitary level. This is supported by Lehnmann *et al.,* (1998) in endurance athletes and Schmikli *et al.,* (2011) who found a blunted cortisol response to exercise in over trained elite middle distance runners and football players. There is also a condition labelled non-functional overreaching (NFO) where training results in no improvement in performance. This is thought to lead to hypersensitivity of glucocorticoid receptors during the second bout of exercise (Meussen *et al.,* 2010). In a recent study they identified that resting cortisol levels were higher in overtrained athletes compared to NFO and there was no increase in cortisol after the first bout of exercise in both overtrained and NFO athletes; however, there was an increase after the second and this was higher in the NFO athletes. It has been proven difficult to distinguish between overtrained and NFO athletes but other hormones such as ACTH and prolactin may prove better in this (Meeusen, 2010). Diagnosis of overtraining syndrome is a growing area and proven criteria could help athletes improve with performance with adequate recovery.

1.9 Summary

This chapter presents a review of steroid hormones, mass spectrometry and detection and quantification of hormones as well as the acute hormonal response to exercise. Cortisol and testosterone are involved in the acute and chronic stress response and measurement in saliva offers a non invasive alternative to blood sampling. Studies of the hormonal stress response to exercise propose a 'threshold intensity' for cortisol and testosterone release during acute exercise; however, findings are contentious and further work is required to confirm this association. Exercise research also includes studies investigating the cortisol and testosterone response to weight training sessions; but aerobic circuit sessions commonly practiced by endurance athletes have been overlooked. Despite studies observing a concomitant increase in salivary measures after intravenous injection, there are mixed reports concerning a time delay of post exercise

steroid hormone peaks in saliva compared to blood. This project aims to bridge some of these identified gaps in exercise research.

Secondly, LC-MS is currently an established gold standard method for analysis of hormones in pharmaceutical, clinical and environmental analytical laboratories however ELISA still remains the primary method used for hormone analysis in exercise research. This review suggests that LC-MS offers an alternative method for analysis of cortisol and testosterone, providing simultaneous quantification of both analytes and high sensitivity and specificity for salivary analysis. Utilising this method may also avoid the potential overestimation of ELISA proposed during quantification of testosterone in women and children. Therefore, the main objectives of this collaborative analytical chemistry and exercise physiology project are to:

- Develop an LC-MS method to detect and quantify cortisol and testosterone simultaneously in saliva;
- Validate the LC-MS method by comparing quantification of resting saliva samples with ELISA;

Following this:

- Use the developed LC-MS method to quantify salivary cortisol and testosterone in response to exercise;
- Investigate the effects of acute training sessions including circuit training on salivary hormone concentrations in endurance athletes;
- Assess the correlation between salivary and blood measures before and after acute exercise.

The thesis is structured in chapters that stand alone, which are presented in a logical order and are linked together to fulfil the main aims of the project.

2. GENERAL METHODS

2.1 Materials

2.1.1 Stocks

For all mass spectrometry optimisation, 50 ng/mL methanolic stocks of dry cortisol and testosterone (Sigma-Aldrich, UK) were prepared weekly by dilution of a 1 mg/mL master stock. The 1 mg/mL stocks contained cortisol and testosterone dissolved in 100% methanol (MeOH) and the stock was stored at -20^{o}C for up to two months, before being discarded and freshly prepared.

2,1.2 Calibration standards

Calibration standards (4mL) were prepared in water at concentrations of 0, 1, 2, 5 and 10 ng/mL of cortisol and 0, 0.1, 0.2, 0.5 and 1.0 ng/mL testosterone. IS (3 ng/mL and 0.3 ng/mL deuterated cortisol (d_2) and testosterone (d_3) respectively; QMX laboratories, Thaxted, UK) was added to all calibration standards. For separate analysis of single analytes, standards were prepared containing only cortisol and cortisol (d_2) IS or only testosterone and testosterone (d_3) IS, the latter was also prepared in the higher range of 0-10 ng/mL (table 2.1). 1000 µL of each stock was used for analysis.

Table 2.1 Calibration standard preparation (4mL)

Stock concentration (ng/mL)		Cortisol stock (µL)	Testosterone stock (µL)	Water (µL)	Internal standard (µL)	
Cortisol	Testosterone				Cortisol	Testosterone
0	0	0	0	3760	120	120
1*	0.1*	400	400	2960	120	120
2*	0.2*	800	800	2160	120	120
5§	0.5§	400	400	2960	120	120
10§	1.0§	800	800	2160	120	120
0		0		3880	120	
1*		400		3360	120	
2*		800		3760	120	
5§		400		3360	120	
10§		800		3760	120	
	0		0	3880		120
	1¶		400	3360		120
	2¶		800	3760		120
	5¶		400	3360		120
	10¶		800	3760		120

Key: * = 10 ng/mL cortisol and/or 1 ng/mL testosterone

§= 50 ng/mL cortisol and/or 5 ng/mL testosterone

¶=10 ng/mL testosterone

2.1.3 Known controls

Known control samples (1000 µL) were also prepared (1.89, 4.72 and 7.54 ng/mL cortisol and 0.19, 0.47 and 0.75 ng/mL testosterone, IS 3 ng/mL and 0.3 ng/mL respectively; table 2.2). Prior to analysis, all samples were diluted with 1000 µL water and underwent solid phase extraction (SPE), after which the eluent was dried and the sample reconstituted in 100 µL 50:50 MeOH:water (concentrating 10 times).

Table 2.2 Known control samples of cortisol and testosterone (1mL)

Stock concentration (ng/mL)		Cortisol stock (10 ng/mL) (µL)	Testosterone stock (1 ng/mL) (µL)	Water (µL)	Internal standard (µL)	
Cortisol	Testosterone				Cortisol	Testosterone
1.89	0.75	200	800	0	30	30
4.72	0.47	500	500	0	30	30
7.54	0.19	800	200	0	30	30
2.00		200	0	770	30	
5.00		500	0	470	30	
8.00		800	0	170	30	
	0.20		200	770		30
	0.50		500	470		30
	0.80		800	170		30

2.2 Analytical methods

2.2.1 LC-MS

The LC-MS instrument was a VG BioQ triple quad LC-MS system (Micromass, UK) with an ESI interface. The system included an Agilent 1200 HPLC system with temperature controlled autosampler (Agilent Technologies, UK). Analytical separation was conducted with a Luna pentafluorophenyl (PFP) phase column (100 x 200 x 3 µm) (Phenomenex, UK). The injection volume was 15 µL and column temperature 25^{o}C. The mobile phase was delivered at a flow rate of 0.15 mL/min with a gradient programme of 15.5 mins (table 2.3); both MeOH and water contained 0.1% formic acid. The MS conditions were optimised for maximal signal intensity for cortisol and testosterone. The machine was operated in positive ion mode with a source temperature of 120^{o}C and desolvation temperature of 300^{o}C. Nitrogen gas flow was 100 L.hr and desolvation gas 350 L.hr. Capillary voltage was 4 kilovolts (kV) and cone voltage 25 volts (V). Low mass (LM) and High mass (HM) resolution were set at 8 Daltons (Da). Scans were performed with SRM for cortisol (*m/z* 363.4), deuterated cortisol (*m/z* 365.4), testosterone (*m/z* 289.4) and deuterated testosterone (*m/z* 292.4). After LC-MS analysis samples were analysed using MassLynx software (version 3.5). Peaks were integrated by conducting a 3 x 2 smooth

automatic integration, identifying the peak area of both analytes and applying manual correction when necessary.

Table 2.3 Gradient and flow rate for LC method

Time (mins)	% A (water)	% B (MeOH)	Flow rate (mL/min)
0	50	50	0.15
2	5	95	0.15
7	5	95	0.15
9	50	50	0.15
15.5	50	50	0.15

2.2.2 Saliva collection and storage

Participants, recuited from the local university and athletics clubs, were instructed to produce at least 2 mL of saliva into a 50 mL centrifuge tube. Parafilm (50 x 100mm) was provided for participants to chew on in order to stimulate saliva production. Participants were instructed to chew on the Parafilm for one minute before emptying their mouths and expectorating any further saliva into the tube with their head tilted forward. Participants continued to chew on the Parafilm during collection to stimulate production. Prior to collection participants were advised to refrain from drinking at least five minutes before producing the sample and from eating or consuming caffeine at least 3 hours before. Samples took between 3 and 10 minutes to produce. Samples were stored for a maximum of 3 months prior to analysis. After production, samples were aliquotted into four 1.5mL Eppendorf tubes, centrifuged at 10,000 x G for 10 mins at room temperature and the supernatant removed and finally put into new Eppendorf tubes and stored at -80^{o}C until analysis. Samples were collected in the morning within 45 minutes of awakening during the resting validation studies and between 3pm and 8pm in the exercise study.

2.2.3 Salivary ELISA analysis

After thawing and centrifugation for 5 minutes at 1500 x G saliva samples (25 µL) were analysed for cortisol and testosterone in duplicate with ELISA (Salimetrics, UK) according to the kit instructions. The calibration curve consisted of five prepared standards (0.012 to 3.0 µg/dL for cortisol and 6.1 to 600 pg/mL for testosterone) and a zero sample. Absorbance values for the unknown samples were interpolated from calibration standards

with a 4-parameter logistic curve (SigmaPlot, version 11). The sensitivity of the kits was 0.03 ng/mL for salivary cortisol and 1 pg/mL for salivary testosterone. For all studies, the mean intra assay coefficients of variation were 8.0% for cortisol and 9.1% testosterone. The mean inter assay coefficients of variation were 7.4% and 5.2 % for cortisol and testosterone, respectively.

2.3 Ethical Approval

Ethical approval was obtained from the University of Greenwich ethics committee for studies in Chapters 5 and 6. All participants were given an information sheet detailing the study and were given the opportunity to ask questions before providing written informed consent (Appendix A). Prior to beginning the study participants completed a health screen questionnaire (Appendix B) and a physical activity questionnaire to assess suitability to take part.

2.4 General statistics

All statistical analysis was performed with SPSS 18.0 software for Windows (SPSS Inc., Champaign, ILL, USA). Data are presented as mean values ± standard deviation (± SD) Data was checked for normality and sphericity prior to statistical analysis. If data was not normally distributed analysis was performed on logarithmic transformed data (Log10). In chapter 3 data is analysed with one way analysis of variance (ANOVA) and independent t-tests. Chapter 4 employs both paired and independent t-tests. Chapter 5 utilises independent t-tests as well as Pearson product-moment correlation coefficient and agreement analysis. In chapter 6 data were analysed with a two way (trial x time) repeated measures ANOVA. Significant differences were assessed with Bonferoni adjustments for multiple comparisons. Additionally, correlations are analysed with Pearson product-moment correlation coefficient and methods were compared with agreement analysis. A p value of < 0.05 was accepted for statistical significance.

3. DEVELOPMENT OF AN LC-MS METHOD FOR THE QUANTIFICATION OF SALIVARY CORTISOL AND TESTOSTERONE

Central to this project is the development of an LC-MS method to quantify cortisol and testosterone in saliva. Chapter 1 identified LC-MS as the gold standard for measurement of small molecules in clinical and pharmaceutical settings and LC-MS was chosen because there is currently an established method to measure salivary cortisol levels with low LOQ (Katoaka *et al.,* 2007) and using a single quad mass spectrometer is more cost effective than tandem mass spectrometry. To achieve this, it is important to optimise various LC and MS parameters. These include MS parameters such as cone voltage, capillary voltage and ion resolution (Jiao *et al.,* 2002). Furthermore LC parameters such as solvent composition and flow rate, pH buffering and column choice and temperature (Dolan, 2010). For application in exercise research an LC-MS method for detection and quantification of salivary cortisol and testosterone levels should:

- Minimise analyte loss during sample preparation
- Involve sample volumes that can easily be collected during and after intense exercise
- Accurately quantify cortisol and testosterone levels in a biological matrix
- Show acceptable intra and inter day precision
- Allow transfer to other laboratories and machines
- Allow fast sample preparation, chromatography and detection
- Be cost effective
- Offer simultaneous detection of multiple analytes

In this chapter, the optimisation of both MS and LC parameter to quantify cortisol and testosterone are presented, along with further ways to improve method sensitivity.

3.1 MS optimisation

Optimisation is conducted to give the highest signal possible to obtain the low levels of detection. This is commonly carried out by infusion of standard solutions directly into the mass spectrometer and manually or automatically tuning paramters (Lehotay, 2005). However, this method fails to consider the effect of actual flow rate or mobile phase

conditions on the mass spectrometer parameters. Additionally, mixtures of analytes are infused and these may have different parameters for optimisation due to differences in polarity and reference concentration (Kruve *et al*., 2010). Low levels of detection required in this method justify linking the LC to MS and coupling of LC to the mass spectrometer during optimisation, ensuring the impact of LC flow rate is considered.

3.1.1 Ionisation

Electrospray ionisation (ESI) conducted at atmospheric pressure can ionise small molecules prior to MS analysis. ESI allows ionisation at lower temperatures than other ionisation methods, such as thermal ionisation and can be used to ionise a wide range of compounds including those that are thermally unstable (Santa *et al.,* 2007). ESI (figure 3.01) is produced by applying a strong electric field (3 kV) to liquid passing through a capillary tube under atmospheric pressure (figure 3.01 A). The electric field is obtained by applying a potential between the capillary and the counter electrode. This field induces a charge accumulation at the liquid surface located at the end of the capillary. The emerging liquid from the narrow capillary adopts the shape of a cone as a result of balance between Colombic repulsion at the surface and the surface tension of the liquid. This is known as the 'Taylor cone' (Taylor, 1964). The ionised droplets pass through heated inert gas (nitrogen), this evaporates the solvent molecules and with this desolvation the droplet size decreases but the charge remains constant. The nitrogen gas is also heated to aid drying of the solvent and the temperature required is optimised depending on the amount of water in the mobile phase and the flow rate. This results in an increase in charge density and electrostatic stress near the surface of a given droplet, when the force of electrostatic repulsion between like charges becomes equal to the surface tension holding the droplet together, the 'Rayleigh limit' (Rayleigh, 1882) is reached. Prior to reaching the Rayleigh limit droplets undergo 'Coulomb fission'; a process which leads to the production of smaller 'offspring' droplets and ultimately gas phase ions.

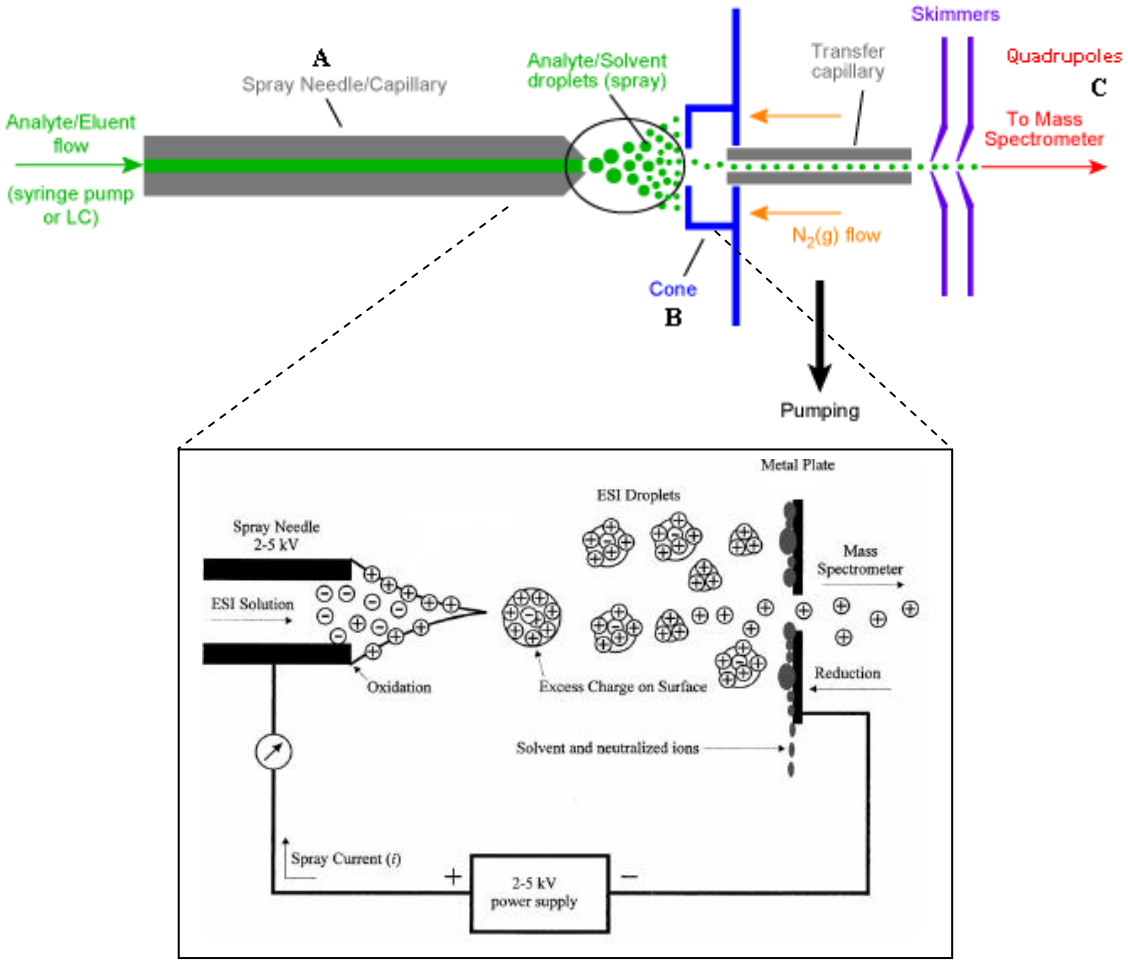


Figure 3.01 Electrospray ionisation coupled to mass spectrometry (University of Bristol, 2010; University of Angers, 2010)

ESI is an ionisation technique that favours multiple charges. Multiply charged ions observed in ESI-MS arise from molecules that have undergone multiple protonations or attachment of several cations (positive ion mode), or have undergone removal of multiple protons or attachment of several anions (negative ion mode) (Cole, 1997). ESI is a soft ionisation technique and as very little residual energy is retained by the analyte upon ionisation and fragmentation is avoided. ESI also favours protonation of ions in the gas phase and cortisol and testosterone observe addition of one charge and an increase in m/z of 1 ($[M+H]^+$). The total number of ions that can be extracted by the mass spectrometer is limited by the electrical current produced by the oxidation or reduction of ions at the probe, therefore ESI is concentration dependent even with a low flow rate (Cole, 2000).

Liquid chromatography factors, including mobile phase composition and additives (Ma and Kim, 1997) play an important role in ionisation and will be discussed later in this chapter. MS parameters also exhibit importance including capillary voltage (which influences ion charge and electrospray efficiency) and cone voltage (influencing transfer of ions to the detector).

3.1.2 Interpreting the mass spectrum

The molecular ion represents the most abundant ion displayed in the mass spectrum (figure 3.03). Mass spectra are dependant on proton affinity and high proton affinity favours the formation of protonated ($[M+H]^+$) ions. Ion selection of steroids commonly occurs in positive ion mode due to protonation of these analytes. Compounds are analysed as their protonated species, although competition between sodium adduct formation and protonation can occur during ESI where $[M+Na]^+$ base peaks are evident (Ma and Kim, 1997). Adduct formation involves a situation where an ion other than a proton forms an adduct with an analyte in solution resulting in a different *m/z* from that of the protonated molecule. Common adducts are formed with sodium, potassium and ammonium and although the exact mechanism for their formation is not clearly understood; however, it is thought that carboxyl or carboxyl ester groups are responsible for binding the alkali metal ions (Lambert, 2004). Sodium and potassium may originate from the biological matrix or glass containers. Alkali metal adducts can reduce signal from $[M+H]^+$ ions and are therefore undesirable (Cole, 1997). During protonation of cortisol and testosterone the proton is most likely to attach the hydroxyl or ketone groups as they are the only functional groups that will ionise in these molecules and proton affinity affects their detection. This is supported by Leinonen *et al.*, (2004) who reported that proton affinity affected ionisation of anabolic steroids in urine, with high proton affinity producing lower detection limits compared to steroids with low proton affinity.

Proton transfer is the most common ionisation reaction, the exact mechanism for ion production is unknown but there are two theories to explain this. Firstly Dole *et al.*, (1968) proposed the charged residue model (CRM) which they hypothesised that as a droplet evaporates, its charge remains unchanged. The droplet's surface tension, ultimately unable to oppose the repulsive forces from the imposed charge, explodes into many smaller droplets. These Coulombic fissions occur until droplets containing a single analyte ion remain. This analyte molecule would retain the ‘residual’ droplet charge as the last solvent

molecules evaporated, thus forming a 'free' gas-phase ion. The second mechanism proposed by Iribarne and Thomson (1976) is named the ion evaporation method (IEM). In this method small droplets form by Colombic fission as in Dole's model; however they proposed that prior to the droplet reaching the Rayleigh limit the charge density on the droplet is sufficiently high to lift a charged analyte molecule, entraining with it a few solvent molecules, from the droplet surface into the ambient gas (Cole, 2000). There is still controversy over which mechanism occurs, although it has been postulated that the CRM occurs with large molecules with masses of at least 3300 Daltons (Cole, 2000), though, there is still debate about desorption of smaller molecules.

Protonation occurs in the liquid phase and ionic modifiers such as formic acid are added to lower the pH of the solution and encourage the molecules to accept a hydrogen ion. Cortisol and testosterone have a PkA >10, therefore addition of an acid will create a liquid with a lower pH and protonation occurs. The choice of mobile phase solvent is also important for MS analysis. Compounds must be dissolved in an appropriate HPLC grade solvent that can be sprayed and also carry the analytes, as well as containing the volatile modifier to maintain pH and promote protonation.

3.1.3 MS parameters

Once appropriate ions have been selected for each compound key MS parameters can be examined to improve ionisation and ion transfer. In single quad MS they are identified as cone, capillary and low mass (LM) and high mass (HM) voltages (Jiao *et al*., 2002).

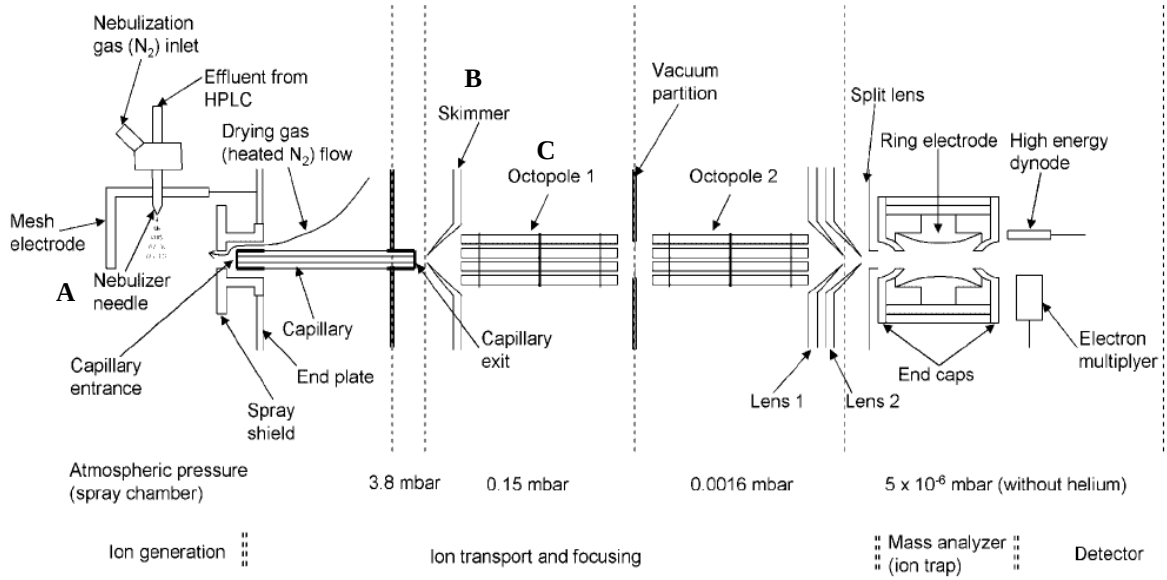


Figure 3.02 Design of an ion trap mass spectrometer to illustrate application of cone voltage (A), Capillary voltage (B) and LM and HM resolution (C) adapted from Kruve *et al.,* (2010).

Cone/skimmer voltage

Cone or counter electrode voltage attracts positively charged ions from the spray into a reduced pressure chamber (figure 3.02 B). If the voltage is low, few ions will be attracted through the skimmer to be focused on the first octopole; however if it is set too high, then a phenomenon known as 'in source fragmentation' can occur. This is where the ions are accelerated into the sprayer at very high speeds and collisions between the ions can fragment them creating smaller ions. This collision is caused by varying the potential difference between the capillary and cone (Wang and Cole, 1997). Insource fragmentation will have a negative effect on signal intensity as the proportion of non fragmented ions reaching the detector decreases. This phenomenon is also difficult to control.

Capillary voltage

Capillary voltage is the voltage applied to the capillary needle, (sometimes referred to as nebuliser needle) in the probe, which leads to the production of charged droplets (figure 3.02A). If the voltage is low the signal intensity will also be low and fewer charged droplets will be created and enter the detector. Adequate voltage applied to the capillary will create a high concentration of charged ions, these will be attracted to the oppositely charged cone and this process of attraction produces the 'Taylor cone' (Taylor, 1964) and subsequent spray. Ultimately, low voltage applied to the capillary will produce insufficient charged ions to form the spray created from the Taylor cone.

HM and LM resolution

HM and LM resolutions are arbitrary values calculated from the RF/DC ratio. The quadrupoles are divides into two pairs of rods. Filtering, or passing a given charged particle along the length of the rod length, is accomplished by applying direct current (DC) and radio frequency (RF) voltages to the rods. Different masses (with associated charge) are affected by changing the RF/DC conditions. The rods are connected as paired opposites with each set alternated as the positive and negative poles by the RF source. The resolutions are applied to quadruple one of a triple quadrupole mass spectrometer (represented by octopole one in figure 3.02C). LM affects the resolution of ions at the low mass range of the quadrupole and HM the high mass range. It is important to optimise these parameters, because if LM and HM are set too low then the isotope peak (carbon 13) will merge with the main peak giving poor mass accuracy; however, if they are set too high over resolution will occur and signal strength will be diminished. Ideal resolution shows the carbon 13 peak without compromising signal strength (Figure 3.03).

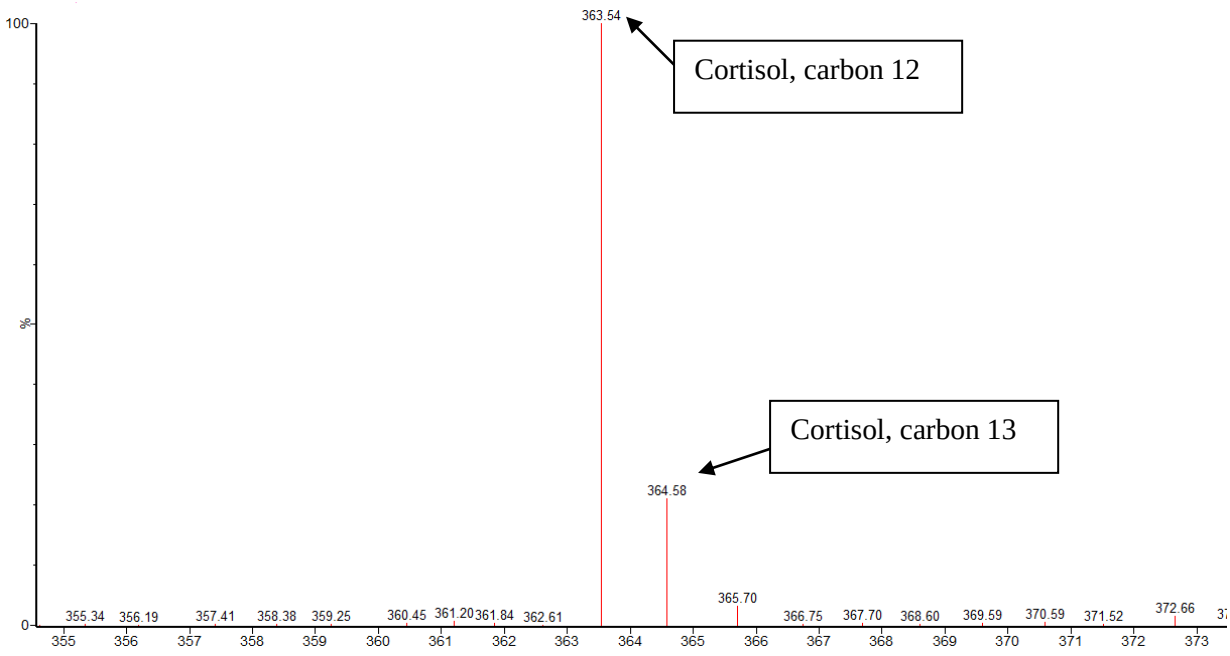


Figure 3.03 Example of cortisol (*m/z* 363.5) showing carbon 12 and carbon 13 peaks

3.14 LC Optimisation

Liquid chromatography separation is based on the selective distribution of analytes between a liquid mobile phase and a stationary phase. The retention time and detection of

analytes can be partly controlled by the chromatography involved in the liquid (mobile) phase and their interaction with the chosen stationary phase (column).

Mobile phase solvent

Mobile phase refers to the composition of the solvent that is used in the HPLC system. Varying mobile phase composition can affect peak shape and intensity as well as retention time (the time it takes for an analyte peak to be detected by the mass spectrometer). In reversed phase chromatography compounds are injected onto a column (commonly C18) and bind to the non polar silica molecules in the column. Less polar compounds will bind more strongly to the silica. Compounds are eluted by reducing the polarity of the mobile phase flowing through the column by adding an organic solvent. When the solvent reaches the same non polarity as the molecules bound to the silica, it competes for binding sites and the analyte is eluted from the column. More polar analytes will be eluted from the column first and exhibit a lower retention time in the resulting mass spectrometer spectrum.

Polarity forms the basis of the rationale for using a ramped gradient for the mobile phase. As the percentage of solvent increase the polarity of the mobile phase will decrease and less polar compounds will be eluted from the column. Gradient elution is especially important in a mixture of compounds with different polarities, as compounds will remain on the column if the percentage of solvent is not increased.

Flow rate

Flow rate is the rate in which the mobile phase is transported through the HPLC system into the mass spectrometer. Flow rate is a key component in Van Deemter's band broadening theory (Van Deemter, 1956). This theory proposes three key elements which effect band broadening; firstly the path an analyte takes through the column packing; secondly, the effect of molecular diffusion; and thirdly, effect of mass transfer between phases. The first two conditions are likely to be influenced by the column packing and dimensions. HPLC columns which contain packing of smaller particle sizes give better efficiency because the diffusion paths are shorter allowing analytes to transfer between the particles more quickly and thus reducing band broadening (Majors, 1973). With relation to flow rate a chromatographic system is in dynamic equilibrium. As the mobile phase is moving continuously, the system has to restore this equilibrium continuously. Since it

takes some time to restore equilibrium (resistance to mass transfer), the concentration profiles of sample components between mobile and stationary phase are always slightly shifted. If the mobile phase flow rate is too high the mobile phase will flow through the column quicker than the analyte, thus leading to peak broadening. A lower flow rate is designed to give a high number of theoretical plates within the column thus providing high column efficiency and narrow peaks; consequently it is important to optimise this LC parameter.

pH

pH can affect the retention time, peak shape and reproducibility of a HPLC method. In cations a decrease in pH can lead to a greater sample ionisation and retention due to its effect on hydrophobicity of an analyte. However, if the addition of ionic modifiers such as formic acid is too high there is likely to be a suppression of MS signal. This is essentially due to an increase in the number of ions in the spray and could decrease access of the target analyte to the droplet surface, eventually leading to complete droplet saturation and suppression of target analyte ionisation (Benijits *et al.*, 2004). It is also important to maintain consistency of the pH in order to reproduce retention times. A buffer such as formic acid can cause ion suppression on the column, which may force molecules to become unionised and therefore increase retention and improve resolution of analytes (Thermo, 2004). It can also lead to suppression of column silanol ionisation (Alexander and Dolan, 2009); finally, a sufficiently low pH can enhance ESI which may improve method sensitivity.

Column type and temperature

The type of column chosen for the HPLC analysis can affect the peak shape and retention time of the analyte peaks. C18 columns are commonly used to measure steroids such as cortisol and testosterone. However, newer columns available claim to give better resolution and peak intensity than the traditional C18; for example the Luna Pentafluorophenylpropyl (PFF) column is designed for increased selectivity of compounds (Havlíková *et al.*, 2008), and the latter column is employed in the current method.

Column temperature can be a powerful tool to increase selectivity and separation of compounds (Dolan, 2002). Previous studies have shown an increase in temperature of 3.75^{o}C was found to have a similar effect on retention as a 1% increase in MeOH

(Bowermaster and McNair, 1984). An increase in temperature lowers the viscosity of the mobile phase and gives molecules more energy moving faster through the column. This can improve column efficiency represented by less band broadening (Vanhoenacker and Sandra, 2006). Manufacturers recommend that column temperature does not exceed 50^{o}C due to concerns of thermal degradation; however, a new generation of silica based phases have recently been developed that are stable up to 200^{o}C (Jones *et al.*, 2005).

3.15 Increasing LC-MS sensitivity

Derivatisation

Changing the chemical structure of a compound can also offer improvements in ionisation and limits of detection. Testosterone is found in low levels in biological fluids, particularly saliva and this provides issues when attempting to quantify extremely low levels found in some women and in children (Kushnir *et al.*, 2006). Therefore, to solve this problem, methods have been sought to dervitise testosterone. Derivatisation of testosterone has been commonly performed using hydroxylamine, this compound reacts with keto groups to form an oxime derivative (Kalhorn *et al.*, 2007). The process for derivatisation of testosterone is detailed it the diagram below (figure 3.04).

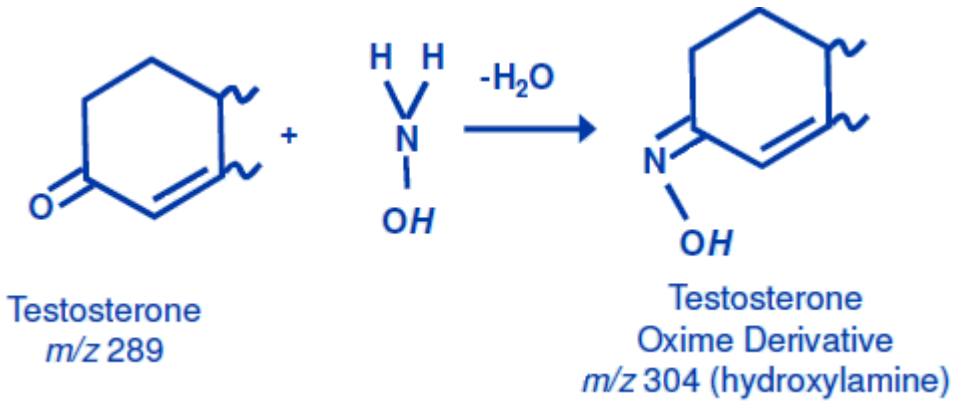


Figure 3.04 Schematic illustrating derivatisation of testosterone (*m/z* 289) with hydroxylamine to form a testosterone oxime derivative (*m/z* 304) adapted from Vesper and Botelho, (2010)

Derivatising compounds has been shown to improve ionisation efficiency and decrease LOD by increasing volatility and decreasing ionisation energy of polar compounds, allowing improved ionisation in the source (Vesper and Botelho, 2010). This work will examine the effect of derivatisation on testosterone in terms of chromatographic separation and LOD.

3.2 Methods

3.2.1 LC-MS

During all analysis a VG BioQ triple quad mass spectrometer (Micromass/Waters) with electro spray ionisation (ESI) interface was coupled an Agilent 1200 HPLC machine (Agilent Technologies UK Ltd, UK). All analysis was undertaken with a Luna PFP column with dimesions 100 x 2mm and 3um particles (Phenomenex Inc, UK).

3.2.2 Stock solutions

A working stock solution containing cortisol (50 ng/mL) and testosterone (50 ng/mL) (Sigma-Aldrich, Gillingham, UK) dissolved in MeOH was used as described in chapter 2.

3.2.3 Ion selection

A full ion scan (*m/z* 50-500 Da) of working stock was performed to identify which ions were most abundant for cortisol and testosterone. Ions chosen were *m/z* 289.4 for testosterone and *m/z* 363.4 for cortisol ($[M+H]^+$) additionally deuterated forms of cortisol (*m/z* 365.4) and testosterone (*m/z* 292.4) (QMX, UK) were analysed for their ion formation.

3.2.4 MS Optimisation

Cone voltage

Cone voltage was manually manipulated (10 to 50 V at 5V intervals, 70% MeOH and 20 to 40 V at 5 V intervals, 90% MeOH) two mobile phases were tested to establish if the results were consistent with changes in MeOH percentage. The working stock solution was analysed for signal intensity, fragmentation, peak area, peak height and signal to noise ratio (S/N).

Capillary voltage

Peak area response of a working cortisol and testosterone solution (50 ng/mL of each analyte) was recorded at various capillary voltages (2 to 5kV, 0.5 kV intervals). Again this was tested with mobile phases of 70% and 90% methanol.

LM/HM resolution

Firstly, peak area response of cortisol and testosterone working stock (Chapter 2) at LM resolution (6-14 Daltons (Da), 1 Da intervals, with HM set at 14 Da) were examined. Secondly, the response of these analytes to varying HM resolutions (6-14 Da; 1 Da intervals) was also tested with the LM resolution set at the established optimum (8 Da). Parameters were assessed using peak area of the cortisol and testosterone stocks (70% and 90% MeOH isocratic mobile phases).

3.2.5 LC method development

Solvent selection

An Acetonitrile (MeCN) and MeOH gradient were tested for its effect on peak area, peak height and S/N ratio for cortisol and testosterone. The gradient employed with a flow rate of 0.3 mL/min is presented in figure 3.05.

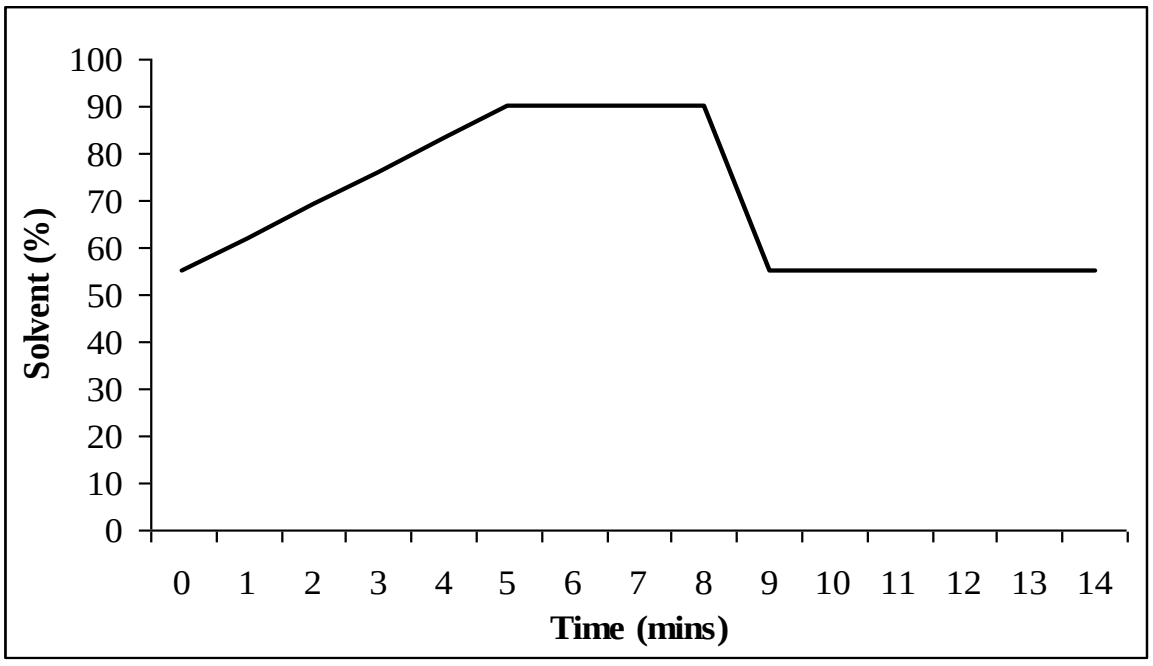


Figure 3.05 Gradient of acetonitrile and methanol for analysis of cortisol and testosterone

Gradient and solvent flow rate

Isocratic (20-90% MeOH) and ramped gradient (table 2.1) methods were assessed for their effect on resolution and signal intensity of cortisol and testosterone (as indicated by retention time, peak area, peak height and S/N of cortisol and testosterone peaks).

Isocratic gradients were tested (flow rates 0.1 mL/min, 0.2 mL/min and 0.3 mL/min) to establish the optimum flow rate. Ramped gradients were initially tested at 0.3 mL/min (peak area, height and S/N ratio of cortisol and testosterone); the best performing gradients in terms of detection sensitivity and resolution were retested at 0.1 mL/min (gradients 1, 2 and 6, table 3.1). The initial gradient was adapted from that employed by Jonsson *et al*., (2003), who measured salivary cortisol and began with 50% MeOH and ramped to 100% MeOH within three minutes. Furthermore, Cawood *et al*., (2005) measured testosterone and eluted with 65% MeOH for three minutes therefore different starting solvents were tested around this range to optimise for cortisol and testosterone. Various solvent percentages were used to begin the gradient in order to optimise run time as well as peak area, height and S/N ratio for both analytes. The gradients below were tested and modification to ramping programmes tested after anlysis of peak characteristics and retention time, with the aim of obtaining clear peak resolution, efficient run time and high S/N ratio.

Table 3.1. %B (MeOH) for 10 ramped gradients

Gradient number	Time (mins)												
	0	1	2	3	4	5	6	7	8	9	10	11	12
1	65	65	65	→	85	85	85	85	85	85	65	65	65
2	55	→	90	90	90	90	90	90	90	55	55	55	55
3	55	→	→	90	90	90	90	90	90	55	55	55	55
4	55	→	→	→	90	90	90	90	→	55	55	55	55
5	55	→	→	→	→	90	90	90	90	55	55	55	55
6	50	→	→	90	90	90	90	90	→	50	50	50	50
7	30	→	90	90	30	30	30	30	30	30	30	30	30
8	70	70	→	→	90	90	90	90	90	70	70	70	70
9	75	75	→	→	90	90	90	90	90	75	75	75	75
10	65	→	→	→	90	90	90	90	90	65	65	65	65

pH

Peak area of cortisol and testosterone (50 ng/mL) were recorded (50-90% methanol, 10 % intervals) at varying mobile phase pH values (2.58 to 3.32) controlled by addition of formic acid (0.1%, 0.2% or 0.5%) added to both MeOH and water of the mobile phase.

Column temperature

Peak area and peak height were recorded for the working stock of cortisol and testosterone (50 ng/mL) for various column temperatures (20-50^{o}C, intervals of 10 ^{o}C, 70% MeOH isocratic mobile phase, 0.3 mL/min).

3.2.6 Chemical modification of testosterone

This method was based on Kushnir *et al*., (2006). Hydroxylamine hydrochloride was obtained from Sigma-Aldrich (Gillingham, UK). Methyl *tert*-butyl ether (MTBE) and HyperSep SPE cartridges were purchased from Fisher Scientific (Loughborough, UK).

Initially, a testosterone stock (1000 ng/mL) was prepared in MeOH (100%). 500 µL stock was evaporated to dryness in a vacuum centrifuge and the dried residue was derivatised with aqueous hydroxylamine solution (300 µL 1.5 mol/L), the samples were vortex mixed and incubated in a heating block (60^{o}C, 5 mins). Water (2 mL) was then added to each tube and the derivatised testosterone was extracted with SPE. HyperSep cartridges (3 mL, 200 mg) were conditioned with MeOH (2 mL) and water (2 mL) then the samples added to the columns. The columns were washed with 20% MeCN (2 mL) and dried (10 minutes), testosterone was then eluted (2x 500 µL MTBE). The solvent was evaporated in a vacuum centrifuge and the residue reconstituted (50 µL mobile phase).

Once this procedure was established to work, lower calibration samples (500 µL) (0, 1, 2, 5 and 10 ng/mL, 3 ng/mL internal standard (d_3 testosterone)) were derivatised, cleaned with SPE and concentrated 10 times to make final concentrations of 10-100 ng/mL. Samples were then analysed (15 µL injection) and S/N ratio compared with non-derivatised testosterone. The above experiment was also repeated with deuterated (d_2) cortisol as an internal standard. Testosterone calibrations and saliva (1 mL) were derivatised with hydroxylamine.

LC-MS analysis was undertaken with an Agilent 1200 HPLC (Agilent Technologies) coupled to a VG BioQ Mass spectrometer (Micromass/Waters) and is described fully in chapter 2.

3.3 Results and discussion

In the following section the optimisation of mass spectrometry settings are discussed followed by liquid chromatography settings and finally observation from the derivatisation of testosterone. During LC-MS analysis it is important to consider all parameters that interact to affect signal intensity and resolution, including adduct formation and ion suppression. The solvent system transporting the analyte into the mass spectrometer can also significantly affect the sensitivity and selectivity of the MS method.

Mass spectrometry optimisation

Each parameter was optimised by assessing the mass spectrum obtained by ESI during LC-MS operation.

3.3.1 Mass spectra

To select precursor ions, full scan (*m/z* range 50-500) mass spectra for each steroid were measured. Results indicate that the most dominant ions are $[M+H]^+$ and $[M+Na]^+$. Sodium $[M+Na]^+$ adduct ions were considered, because as well as being contained in saliva, sodium is a common impurity in MS sample analysis (Lambert, 2004). After a full scan of a combined (50 ng/mL) cortisol and testosterone stock, the ions below were found to be most abundant for the respective analytes (figure 3.06, 3.07). The lower values represent the $[M+H]^+$ peaks (*m/z* 289 and 363) and the higher values represent the sodium adduct ion (*m/z* 311 and 385). The more abundant protonated ions were chosen for analysis.

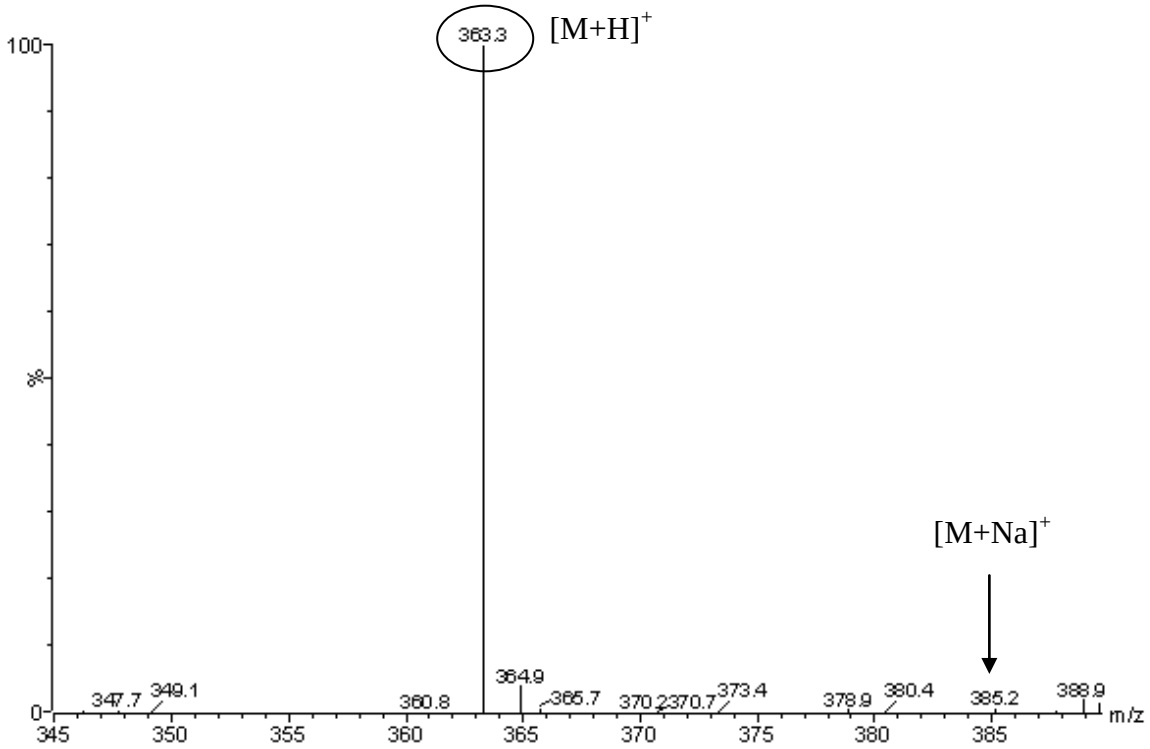


Figure 3.06 Cortisol ion (*m/z* 363.3) and sodium adduct (*m/z* 385.2)

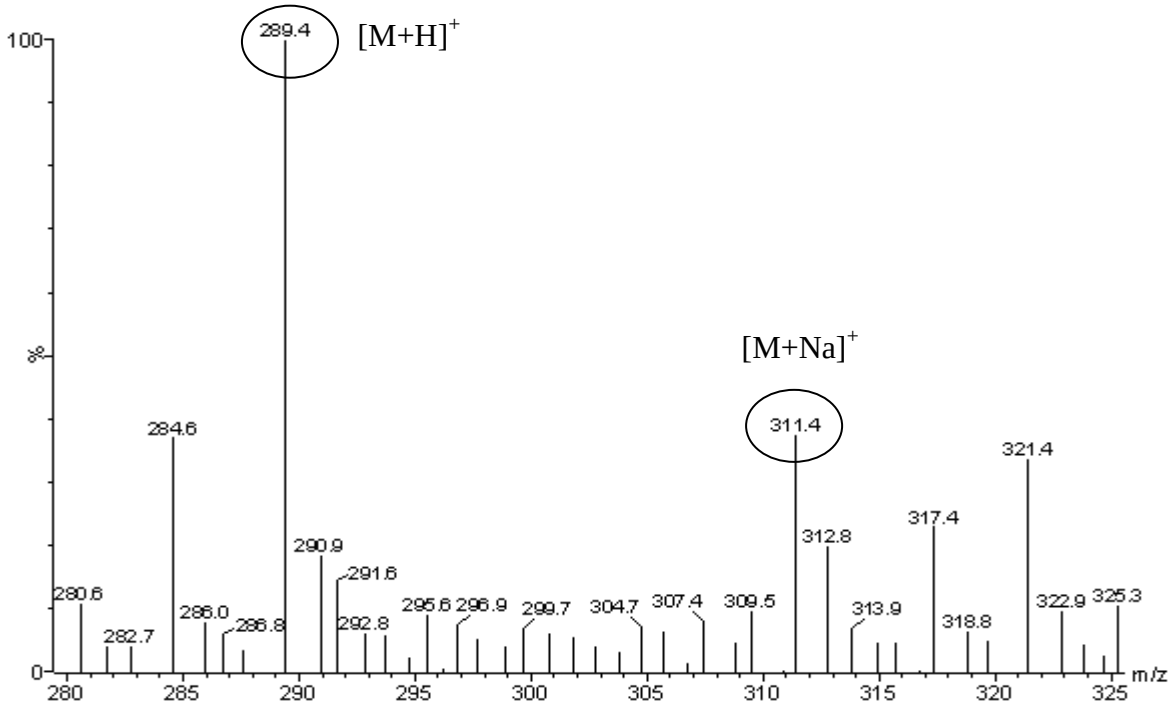


Figure 3.07 Testosterone ion (*m/z* 289.4) and sodium adduct (*m/z* 311.4)

When quantifying the analytes, two deuterated internal standards were added to the samples (fig 3.08 d_3 testosterone, m/z 292.4 and fig 3.09 d_2 cortisol, m/z 365.2). Deuterated internal standards were chosen for their similar physicochemical properties to

undeuterated forms of cortisol and testosterone and to control for fluctuations in ionisation. Similarly the protonated ($[M+H]^+$) ions were most abundant.

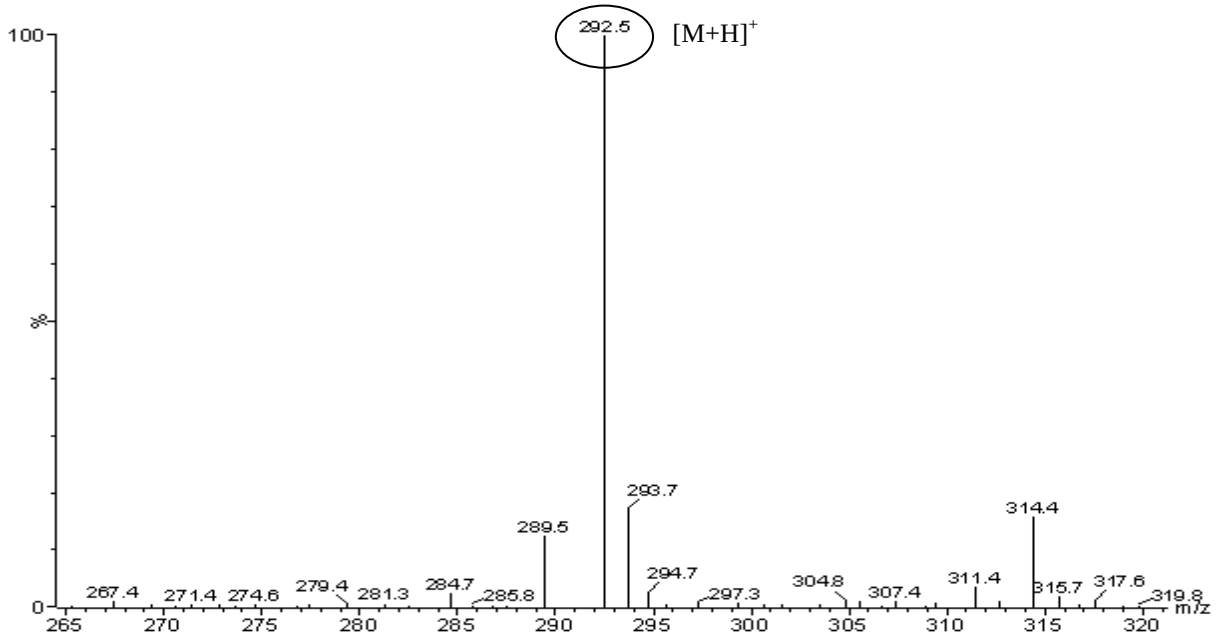


Figure 3.08 Deuterated testosterone ion (m/z 292.5)

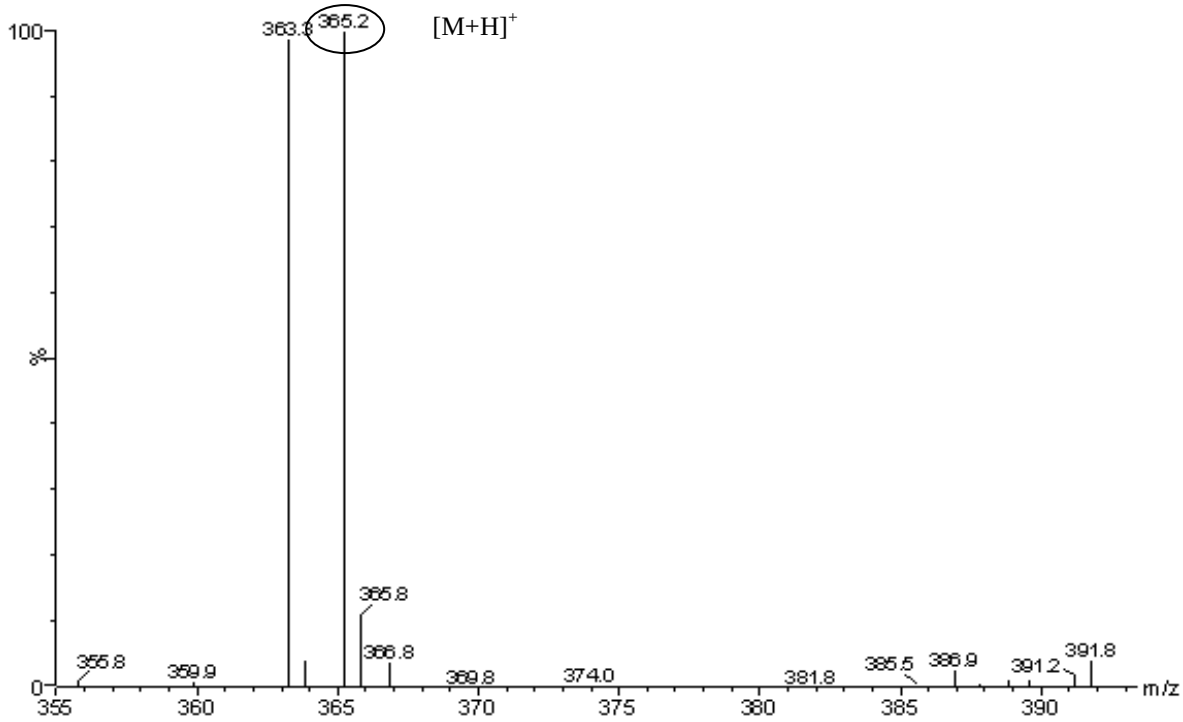


Figure 3.09 Deuterated cortisol ion (*m/z* 365.2)

3.2.2 Cone voltage

Results illustrate that a cone voltage of 30 V for testosterone and 25 V for cortisol produced the highest peak areas. For testosterone, as the cone voltage increases from 10 V

to 30 V there is an almost linear increase in the observed peak area, corresponding to a 130% increase in testosterone peak area. This increase is followed by a rapid linear drop from 30 V to 40 V. The increase in testosterone peak area is caused by an improved ion transmission through the sample cone (Murata *et al.*, 1994); whereas the decrease in peak area is caused by the uncontrolled fragmentation of the pseudo molecular ion, which is confirmed by the increase of in source fragment production. As the cone voltage increases a constant increase in number of fragments formed in the source is observed (figure 3.10). For cortisol, as cone voltage increases from 10 V to 25 V, a 134% increase in peak area is observed. This is followed by a linear decline between 25 V and 40 V. This increase is also due to improved ion transmission and the decrease is caused by an increase of in source fragmentation which occurs after 25 V.

The study was repeated with a mobile phase consisting of 90% MeOH. Peak area was highest at 25 V for both cortisol and testosterone and the number of fragments lowest at the same voltage for both analytes (figure 3.11). There was an increase in testosterone peak area between 10 V and 25 V of 158% and a linear decline from 25 V to 40 V. This coincided with a stable number of in source fragments between 10 and 25 V, followed by an increase from 25 V to 40 V, representing the increased ion energy causing collisions and fragmentation in the vacuum interface. Cortisol showed an identical trend with peak area, increasing from 10 V to 25 V by 122%, then declining to 40 V and fragmentation increased between 25 V and 40 V.

Peak area for cortisol and testosterone is higher in the second experiment, employing 90% MeOH, this may have been due to reduction in background noise levels of the mass spectrometer or improved ionisation efficiency, due to the higher percentage of solvent leading to more efficient evaporation and smaller droplets (Smith *et al*., 2002). The relative percentages of aqueous and organic solvents will affect the signal intensity in MS, due to their effect on the performance of the vacuum interface (Watson and Sparkman, 2007). In ESI the higher the organic content, the more rapid and complete desolvation will occur and therefore there will be increased ion formation and higher signal strength.

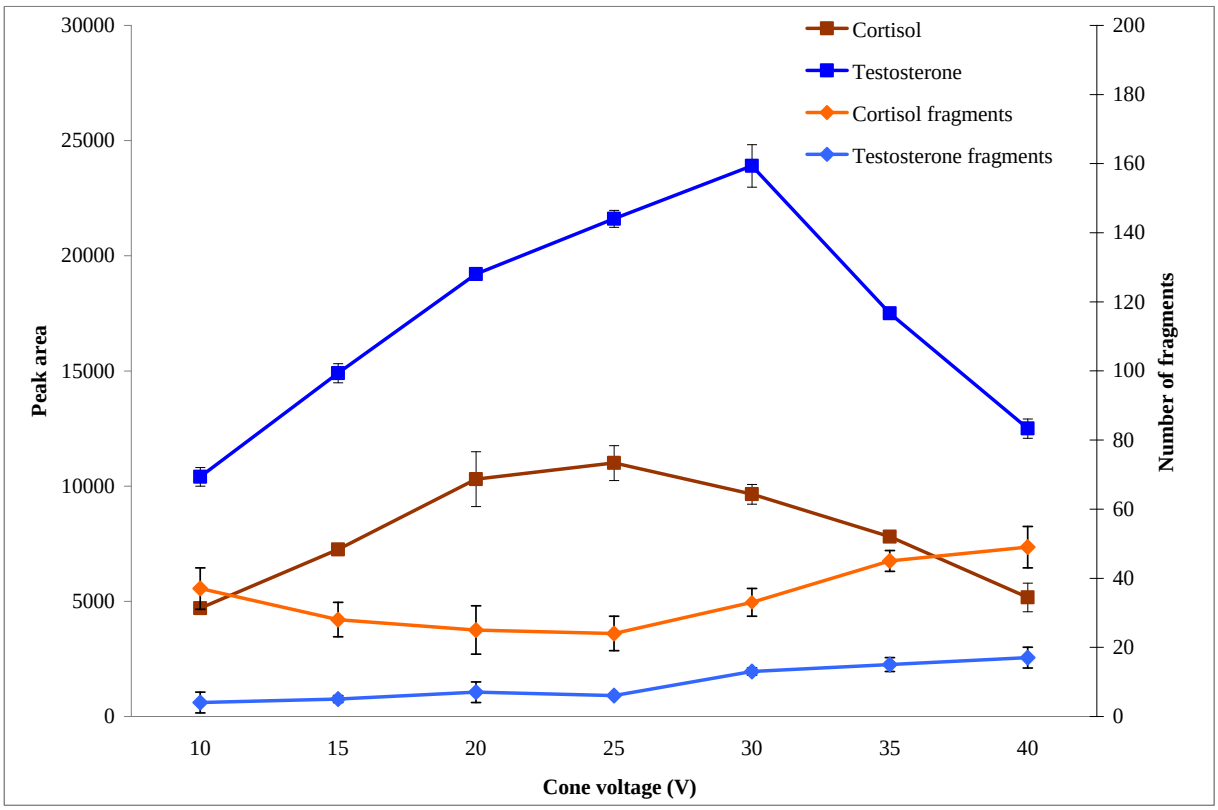


Figure 3.10 Peak area and number of fragments (± SD) (n=4) observed in the mass spectra of cortisol and testosterone collected at cone voltages 10-40 V with 70%v/v MeOH mobile phase

Comparison of cortisol and testosterone reveals a higher peak area for testosterone and this may be linked to a 'hydrophobicity effect' (Marmur *et al*., 2000), which occurs during ESI. It has been shown that more hydrophobic compounds elicit greater signal intensity (Null *et al*., 2003). Testosterone is more hydrophobic than cortisol and therefore moves to the edge of the droplets in electrospray to compete for charges. In turn, a higher signal is observed for testosterone as a higher proportion of droplets are charged and are detected by the mass spectrometer. This is hypothesised to be due to more hydrophobic compounds migrating towards the inter droplet air space and therefore competing better for charges on the droplet surface (Abaye *et al*., 2011).

A cone voltage of 25 V was chosen for this method to provide minimal fragmentation and highest peak area for cortisol and testosterone. This voltage maximises the proportion of ions entering the detector, contributing to a higher detector response.

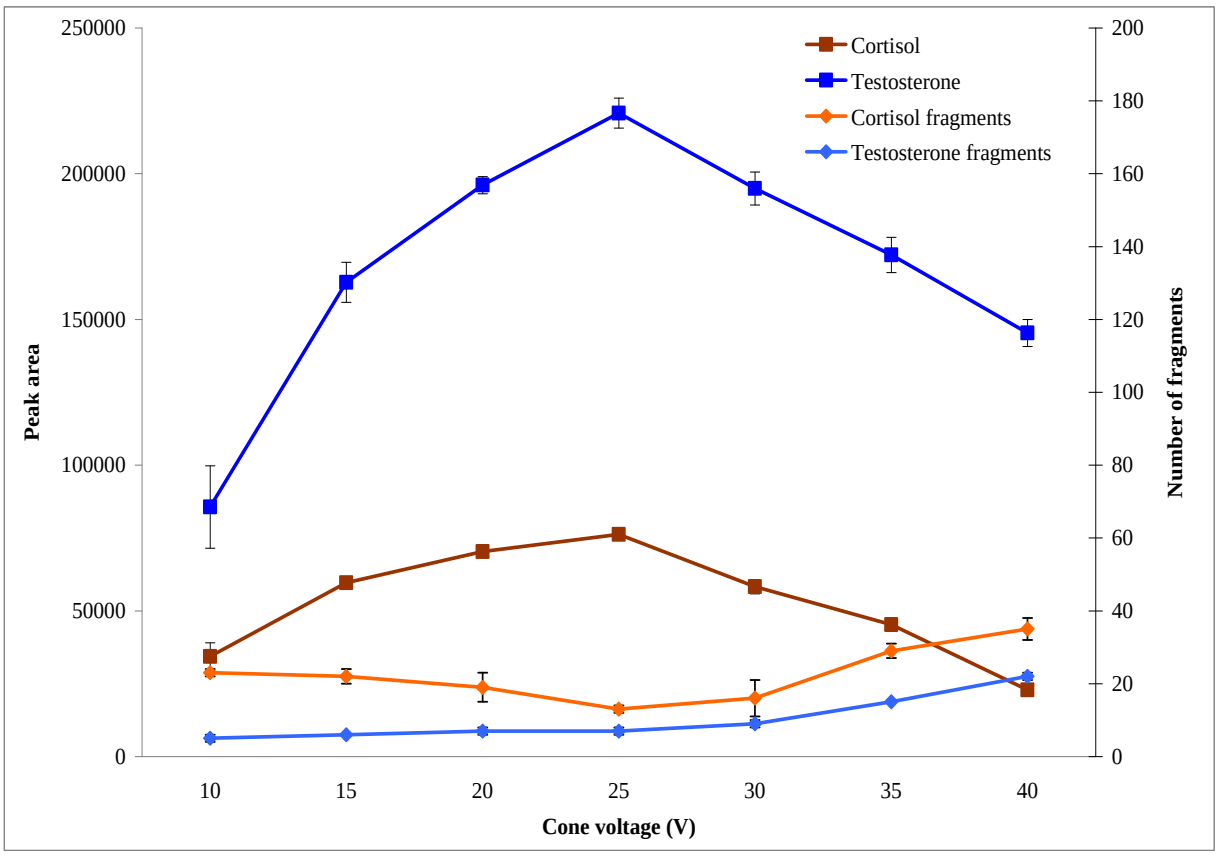


Figure 3.11 Peak area and number of fragments (± SD) (n=4) observed in the mass spectra of cortisol and testosterone collected at cone voltages 10-40 V with 90%v/v MeOH mobile phase

3.3.3 Capillary voltage

In this study peak area of cortisol and testosterone were measured at capillary voltages of 2-5 kV (figure 3.12). Cortisol peak area showed a linear increase between 2.0 kV and 5 kV representing an increase of 33%. There was also an increase in peak area from 3.5 kV to 5 kV of 9%. Peak area was higher at 2.5 kV compared to 5.0 kV for testosterone equating to a 5.3% increase, therefore intra day machine fluctuations negate this difference. No difference was observed for testosterone peak area between 3.5 kV and 5 kV.

The study was also repeated with a 90% MeOH mobile phase (figure 3.13) and for cortisol peak area was higher at 3.0 kV, 3.5 kV, 4.0 kV and 4.5 kV compared to 5kV (12.8%, 16.1%, 15.8% and 11.1% respectively). The largest peak area was observed at 3.5 kV. For testosterone, the highest peak area was also reported at 3.5 kV; however this was not significantly higher than the peak area produced by 3.0 kV. Evaluation of the results concluded the optimum capillary voltage was 3.5 kV as it produced the highest peak area

for cortisol and testosterone in experiment two and in experiment one although cortisol peak area at 3.5 kV only differed from 5 kV by 9% and there was no difference between capillary voltages for peak area of testosterone. These results are supported by the recommendation of 3.5 kV as optimum for LC-MS analysis (Waters, 2011).

Low capillary voltage prevents the formation of spray during ESI and insufficient charge will reduce the number of ions reaching the counter ion (cone). This may explain the linear increase in cortisol peak area between 2.5 KV and 5 kV in experiment one. If there is a sufficient voltage gradient between the capillary and cone this process of attraction produces the 'Taylor cone' (Taylor, 1964) and once the Rayleigh limit (Rayleigh, 1882) is reached, Coulomb fission occurs and the cone splits to form small charged droplets. With a low voltage gradient there will be insufficient charge to form the Taylor cone or to draw ions into the detector.

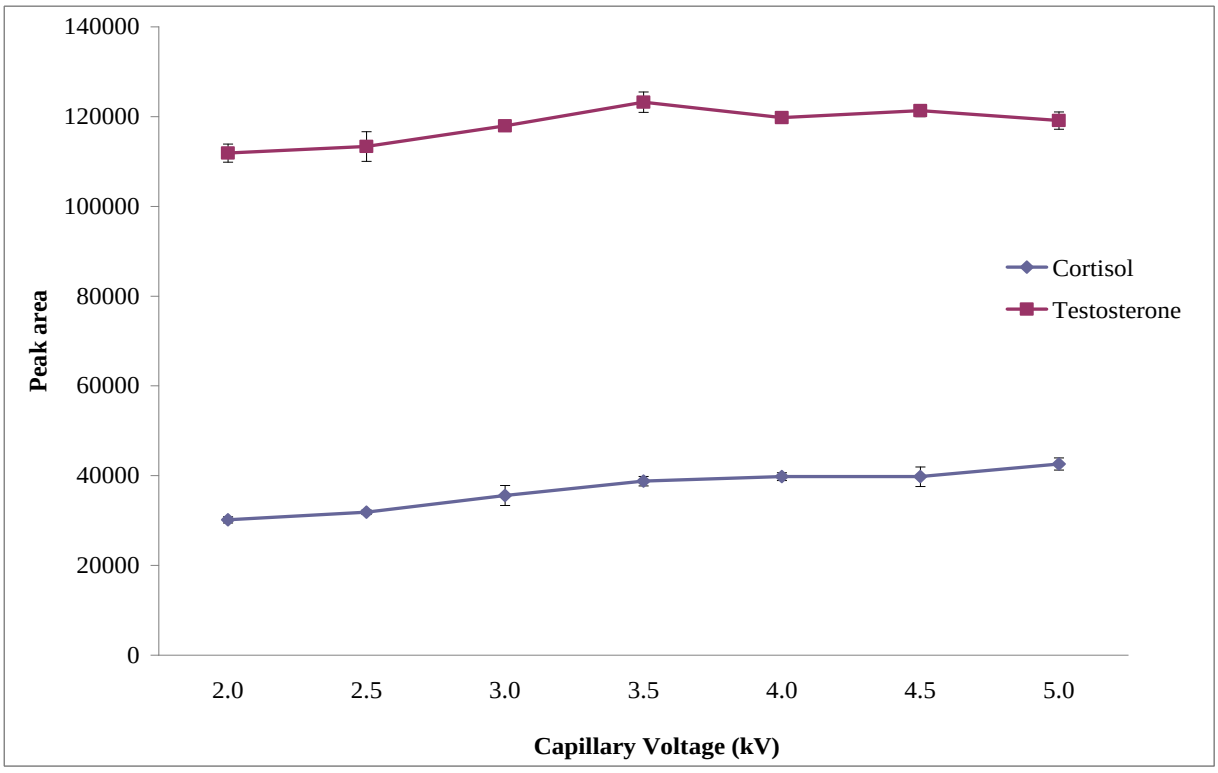


Figure 3.12 Peak area (± SD) (n=4) observed in the mass spectra of cortisol and testosterone collected at capillary voltages 2-5 kV with 70%v/v MeOH mobile phase

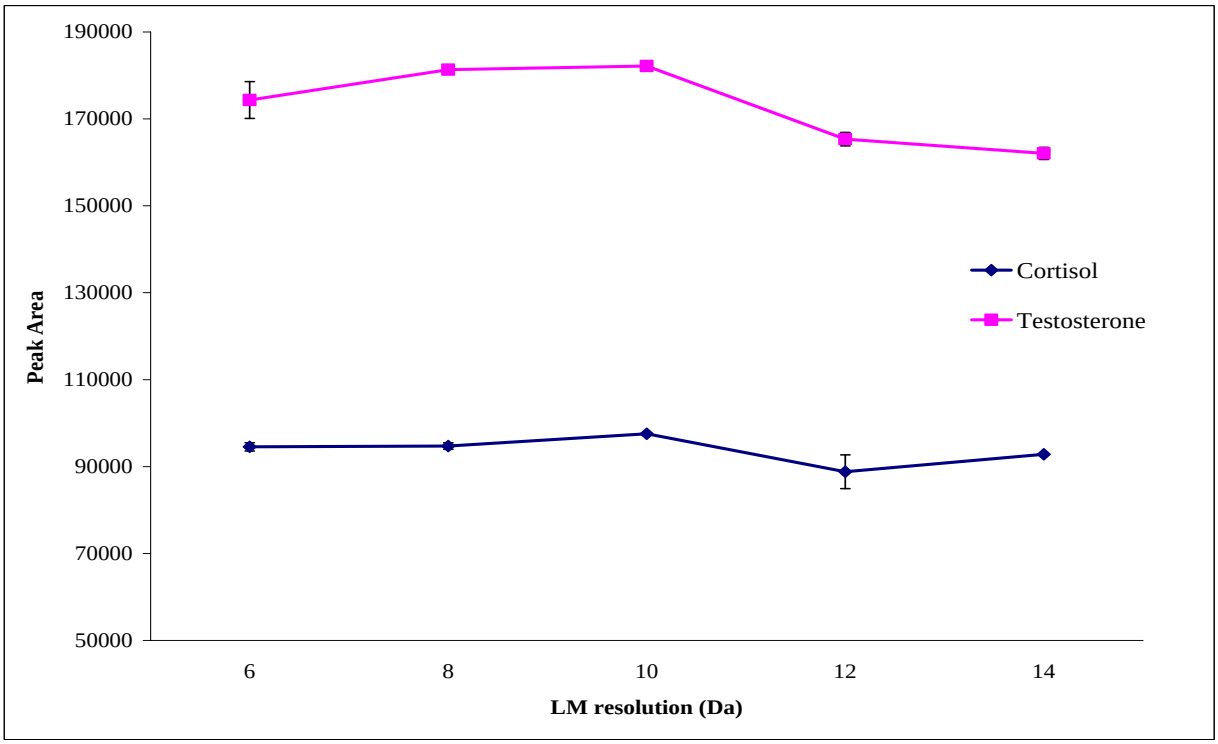


Figure 3.13 Peak area (± SD) (n=4 observed in the mass spectra of cortisol and testosterone collected at capillary voltages 2-5 kV with 90%v/v MeOH mobile phase

3.3.4 Low mass (LM) and high mass resolution (HM)

LM was examined from 6-14 Da in two Da increments with HM resolution set at 14 Da (70% MeOH mobile phase) (figure 3.14). For cortisol, 12 Da produced lower peak area than all other resolutions and this equated to 9.8% difference. Testosterone showed a higher peak area at a LM resolution of 10 Da and this was higher than 12 Da, however 8 Da did not differ (Figure 3.15). This experiment was repeated with a second mobile phase (90% MeOH), and LM resolution of 6 Da for cortisol and testosterone elicited the highest peak area. In conclusion, 8 Da was chosen for LM resolution as it consistently elicited a higher PA.

HM resolution was also examined from 6-14 Da, with LM resolution set to 8 Da. Employing a 70% mobile phase (figure 3.16) results revealed there was no difference in cortisol PA between 6, 8 and 10 Da, though 8 Da was significantly higher than 12 and 14 Da (>18%) ($p=0.0001$). Testosterone peak area exhibited a similar pattern with 8 and 10 Da higher than 12 and 14 Da (<20%). This experiment was repeated with 90% MeOH (figure 3.17), highest peak area was at 6 Da for cortisol (>12%) ($p=0.001$) and the same voltage for testosterone, which was also reported >12% higher peak area than the next

closest resolution. In conclusion, 8 Da was chosen for HM resolution, as it consistently elicited a high peak area in both experiments.

LM and HM resolutions affect the resolution of low mass ions and high mass ions on the quadrupole. If the LM and HM are set too low, the quadrupole acts as a transmission cell and the carbon 13 isotope peak (1.1% of carbon ions) will merge with the main peak giving poor mass accuracy. If the resolution is high, the peaks may become over-resolved therefore compromising sensitivity; this exemplifies the importance of optimising resolution appropriately (Mallet *et al.*, 2004). Addionally, examination of resultion at 8 Da revealed sufficient peak resolution. An example of the ion spectra, illustrating the carbon 13 peak for cortisol is presented in figure 3.03.

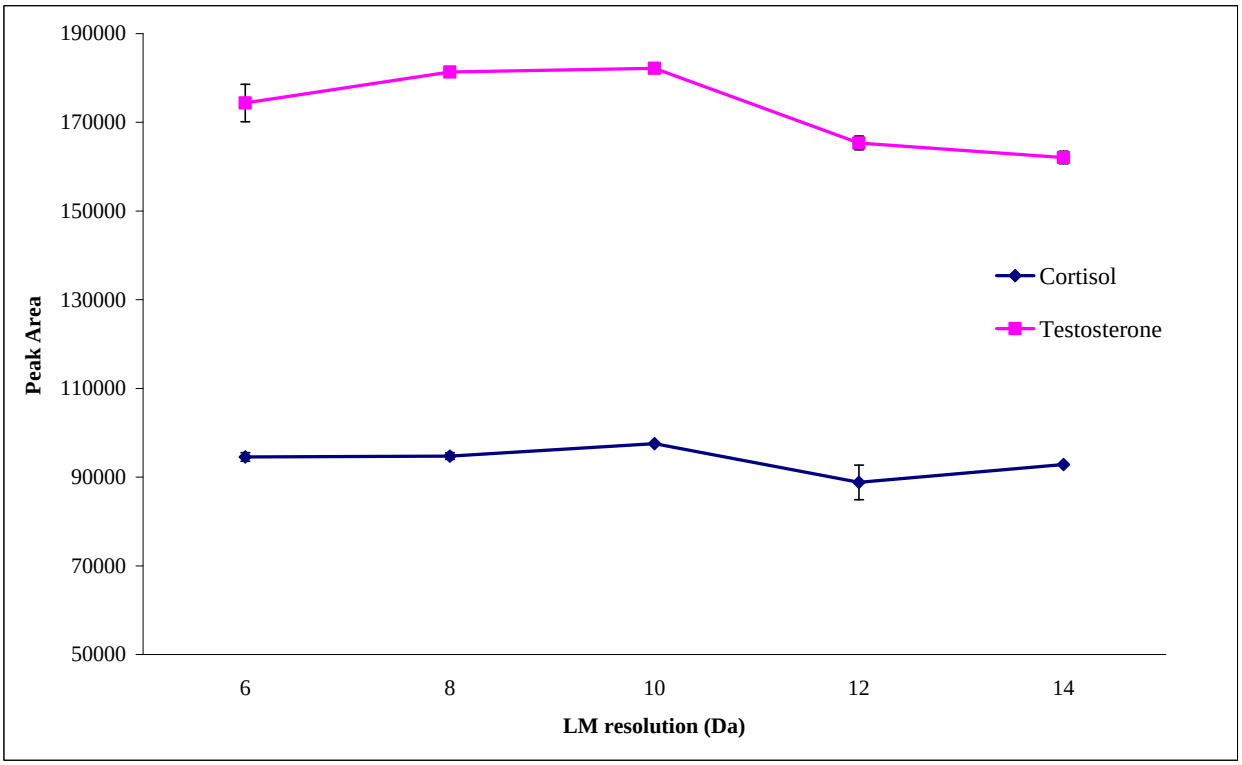


Figure 3.14 Peak area (± SD) (n=4) observed in the mass spectra of cortisol and testosterone collected at LM resolution of 6-14 Da, HM resolution of 14 Da, with 70%v/v MeOH mobile phase

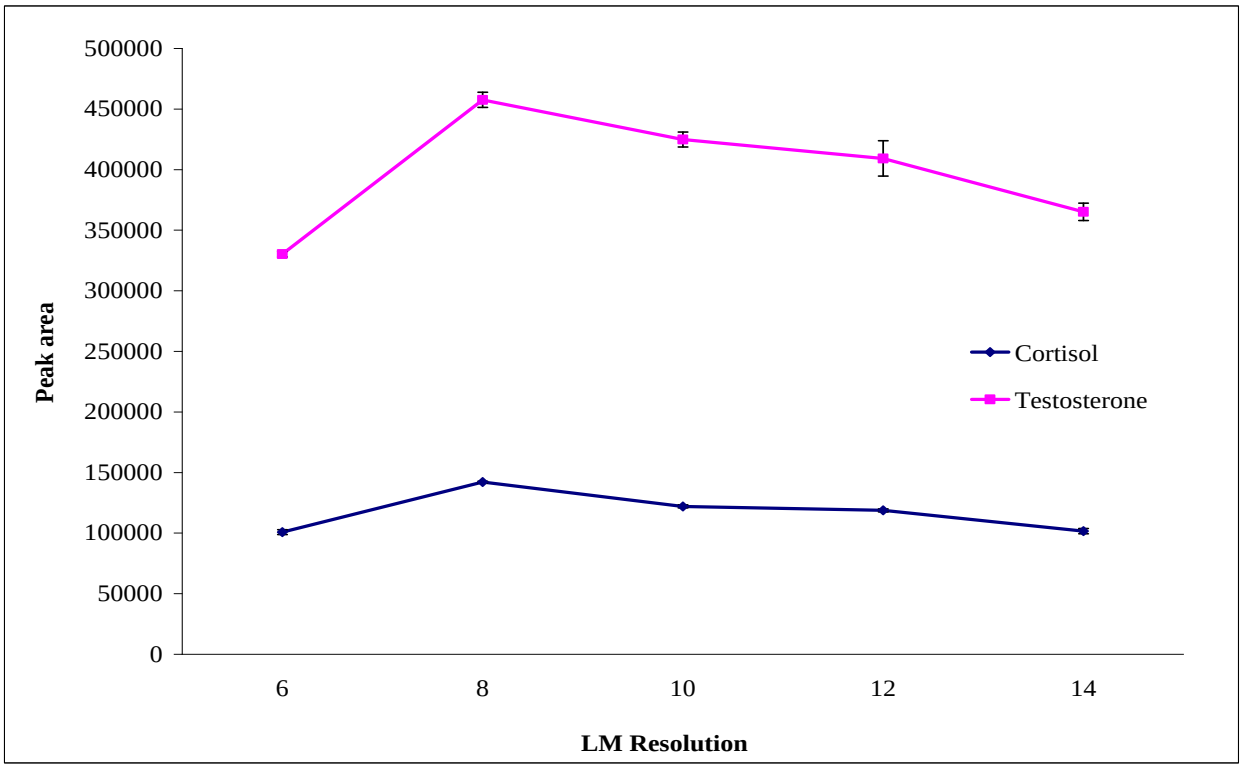


Figure 3.15 Peak area (± SD) (n=4) observed in the mass spectra of cortisol and testosterone collected at LM resolution of 6-14 Da, HM resolution of 14 Da, with 90%v/v MeOH mobile phase

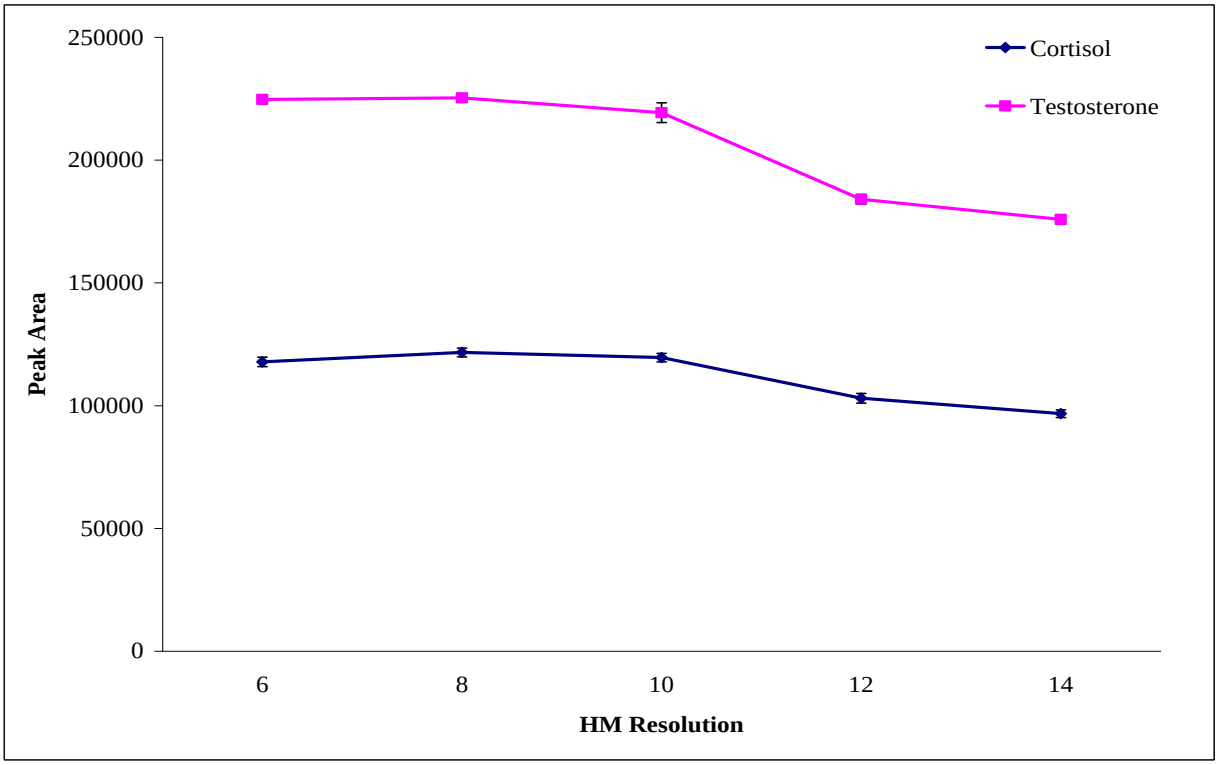


Figure 3.16 Peak area (± SD) (n=4) observed in the mass spectra of cortisol and testosterone collected at HM resolution of 6-14 Da , LM set at 8 Da with 70%v/v MeOH mobile phase

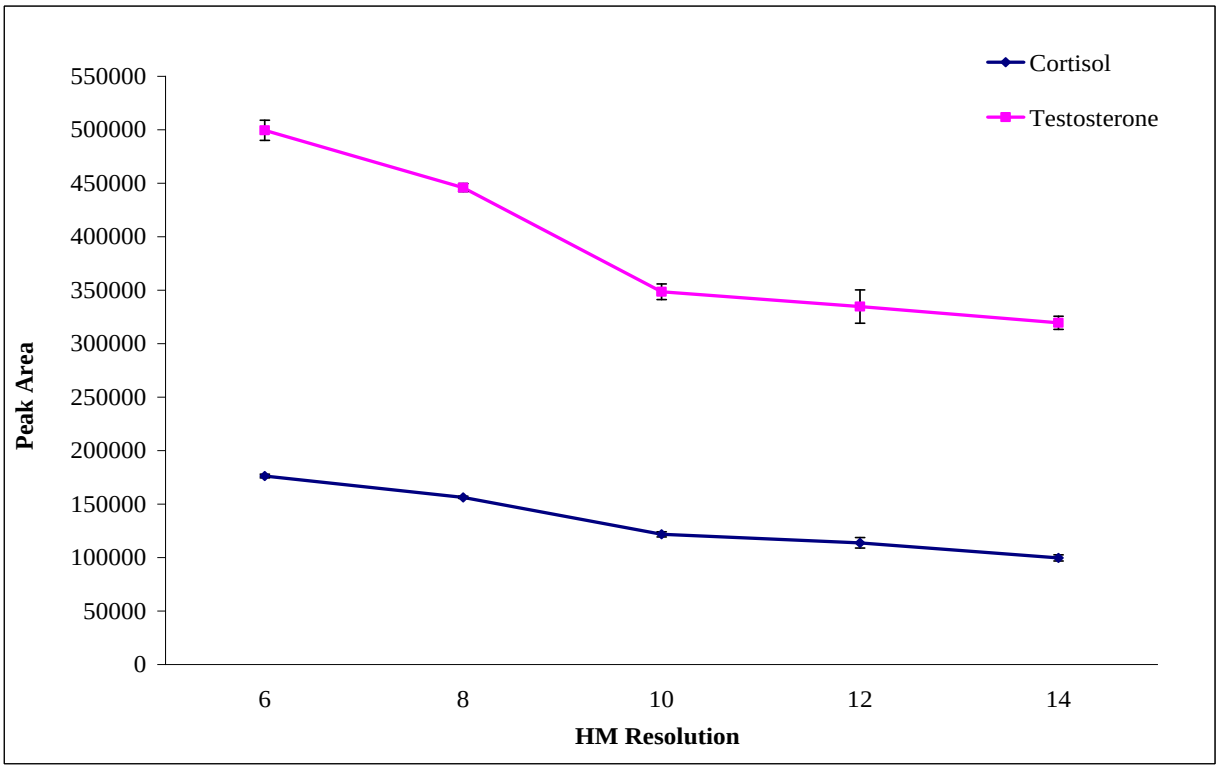


Figure 3.17 Peak area (± SD) (n=4)) observed in the mass spectra of cortisol and testosterone collected at HM resolution of 6-14 Da, LM resolution of 8 Da, 90%v/v MeOH mobile phase

Optimisation of MS parameters serves to increase ionisation and transmission of ions to the detector, as well as improve resolution and mass accuracy (Jiao *et al*., 2002). As previously discussed, in addition to MS optimisation, it is also possible to increase ionisation and in turn lower limits of quantification, through optimisation of LC parameters.

LC Optimisation

3.3.5 Mobile phase solvent

Mobile phase solvent choice is important in LC-MS as it can influence ionisation of analytes and peak retention, therefore it is important to choose the most appropriate solvent for each method. MeCN and MeOH mixed with water were investigated for their impact on peak area of cortisol and testosterone. Examination of the effect of MeCN compared to MeOH on peak area found that for cortisol, a mobile phase containing MeOH elicited 29% higher peak area than the same mobile phase using MeCN. Similarly, for testosterone the peak area was 30% higher will MeOH compared to MeCN. This is illustrated by presentation of different chromatograms and peak areas in figure 3.18.

Some LC-MS methods use MeCN as a mobile phase solvent, rather than MeOH as it is less viscous, resulting in a lower column back pressure thus allowing faster flow rates and it is used as a solvent in hormone measurement research (Singh, 2008). This work supports this observation revealing that peak retention times for cortisol and testosterone were shorter when using MeCN (figure 3.18). This is due to higher polarity of MeCN, leading to faster movement through the column, and when mixed with water MeCN possesses greater elution strength than MeOH (Shimazdu, 2010). These solvents also have different chemical properties, as MeOH is protic (capable of hydrogen bonding) and MeCN is aprotic (not capable of hydrogen bonding).

There is also evidence to suggest MeOH produces significantly higher ionisation of some steroids than acetonitrile (Ma and Kim, 1997). However, this study utlised atmospheric-pressure chemical ionisation and results may not be transferrable to ESI. Furthermore due to lower viscosity, MeCN elicits lower backpressure than MeOH, although in this method backpressure was not an issue. Finally, during the initial period of method development there was a shortage of MeCN and prices increased dramatically (Tullo, 2008) further justifying the use of MeOH in this method.

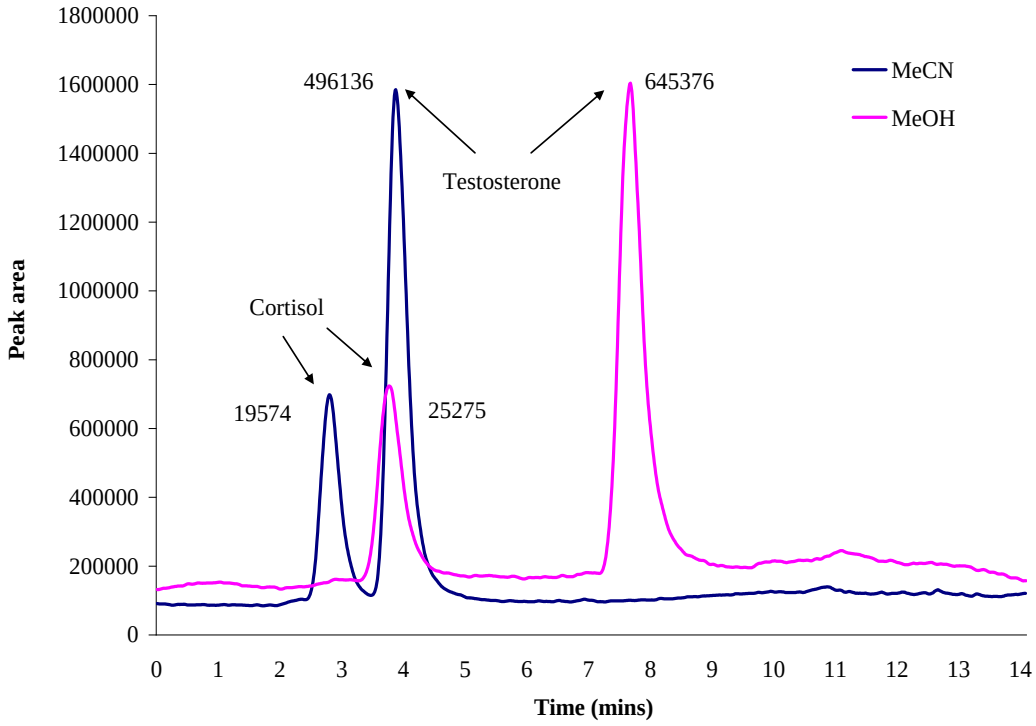


Figure 3.18. Peak area of cortisol and testosterone (50 ng/mL) with comparison of MeOH versus MeCN gradient

Another observation is that cortisol elutes before testosterone as it is more polar and spends less time interacting with the column bonded phase. This leads to differences in peak retention and shape and it is important to consider the chromatographic behaviour of both analytes when optimising the LC method.

3.3.6 Solvent flow rate and isocratic gradients

Firstly, the effect of flow rate on peak area was examined for cortisol (figure 3.19) and testosterone (figure 3.20). There was no change in peak area for cortisol or testosterone from 50-80% MeOH but there is an increase at 90% MeOH. The effect of flow rate was examined for peak height, to investigate the effect of flow rate and organic solvent percentage on peak broadening. Peak height was higher for both cortisol and testosterone at 0.1 mL/min compared to 0.2 mL/min and 0.3 mL/min (figures 3.21 and 3.22). Similarly, there was also a linear increase in peak height with an increase in MeOH concentration. For cortisol, at 0.1 mL/min peak height increased 455% between 50 % and 90 % MeOH. For testosterone the peak height increased 480% between 60 % and 90 % MeOH.

These results suggest taller narrower peaks are produced with an increase in solvent percentage. This may be due to improved ionisation efficiency with a flow rate of 0.1 mL/min. At the liquid flow rates of conventional LC separations, ESI-MS response typically appears concentration-sensitive rather than mass-sensitive; that is, increasing the flow rate does not greatly increase the signal (Bruins, 1991). However, as flow rates are lowered, smaller charged droplets generated by the electospray results in increased ionisation efficiency (i.e. transfer of an ion from solution to the gas phase) and also permit the ESI emitter to be positioned closer to the MS inlet, allowing more efficient transport to the MS analyser, both of which provide increased sensitivity (Gale and Smith, 1993; Wilm and Mann, 1994) (ESI, figure 3.01). The column dimensions were 100 x 2mm with 3 µm particle size and given the particle size the suggested flow rate is 0.1 mL/min for optimal performance (Phenomenex, 2010). This flow rate is designed to give a high number of theoretical plates within the column, thus providing high column efficiency and narrow peaks (Van Deemter, 1956). Smaller inlet diameter columns with low flow rates have been shown to produce higher sensitivity than larger internal diameter (ID) columns with higher flow rates (Shen *et al.*, 2002). This is because peak volume (peak width in

volumetric terms), declines with the reduction of the cross-sectional area of the column, or square of the change in diameter and translating into proportionally taller peaks (Dolan, 2010). Formation of narrower peaks with a higher percentage of MeOH are likely to be caused by a reduced viscosity, leading to less peak broadening prior to detection

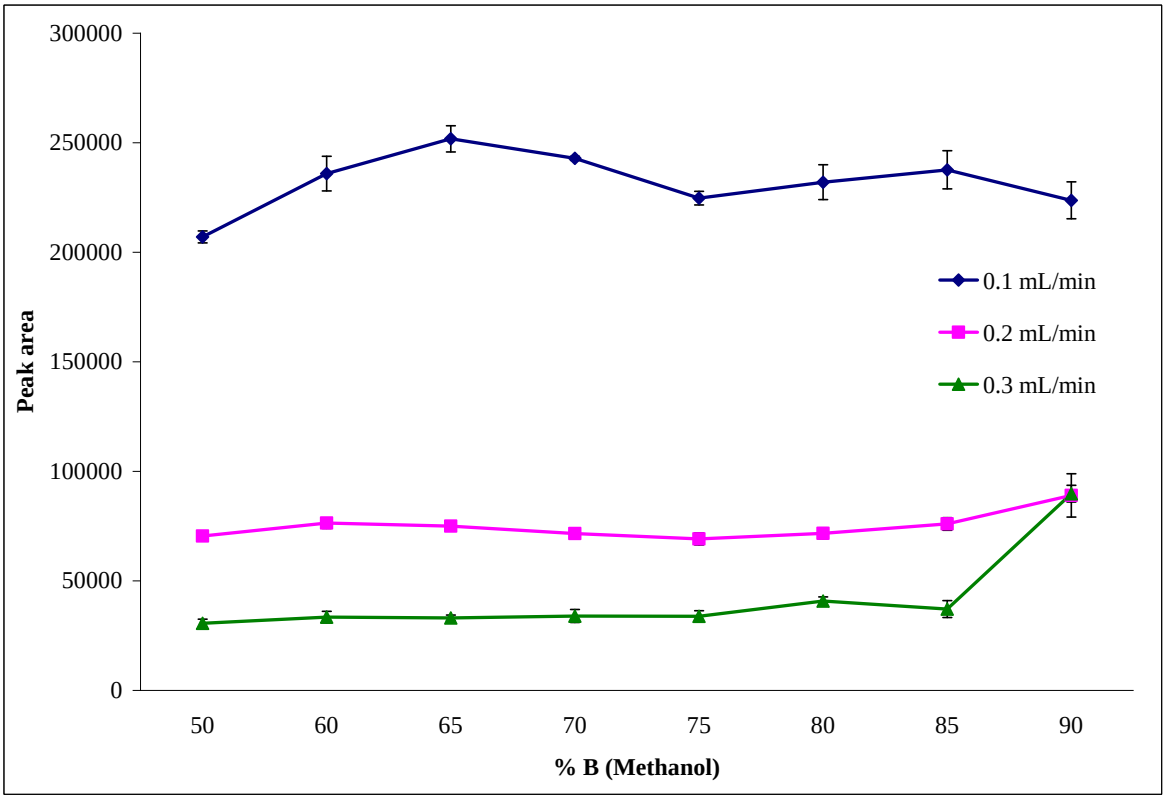


Figure 3.19 Peak area (± SD) (n=4) observed in the mass spectra of cortisol collected at at 50-90%v/v MeOH and flow rates 0.1-0.3 mL/min

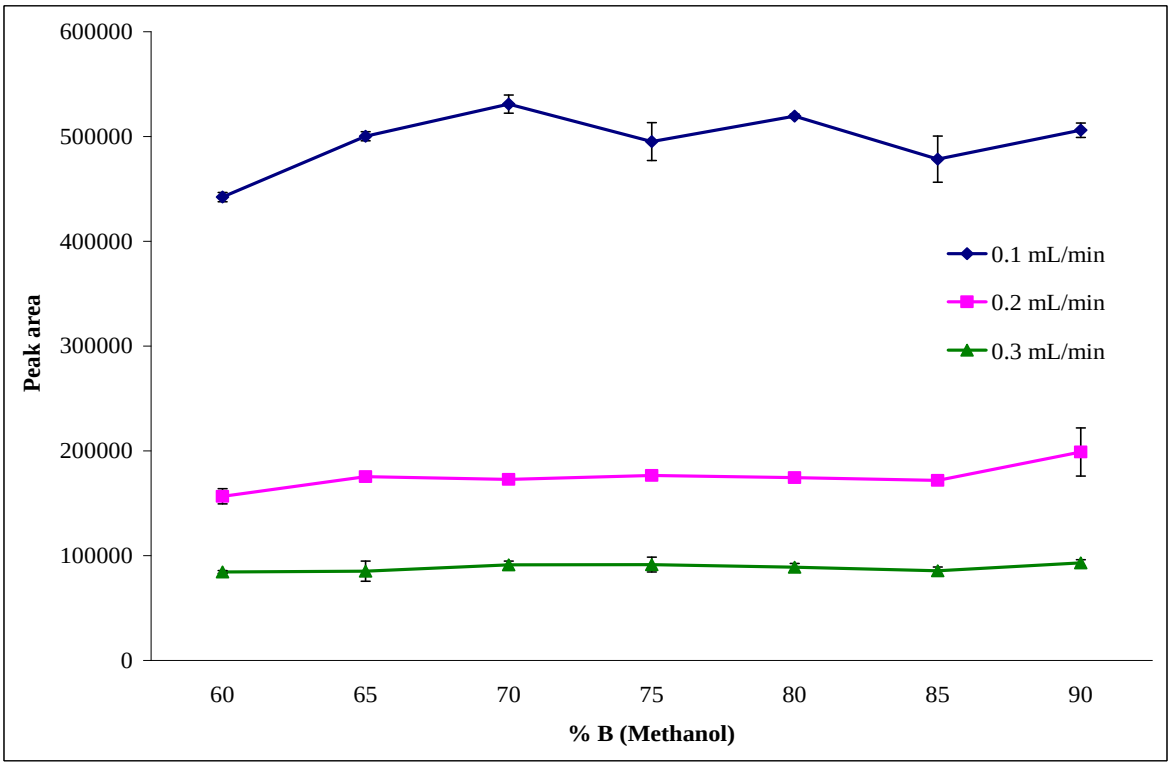


Figure 3.20 Peak area (± SD) (n=4) observed in the mass spectra of testosterone collected at 50-90%v/v MeOH and flow rates 0.1-0.3 mL/min

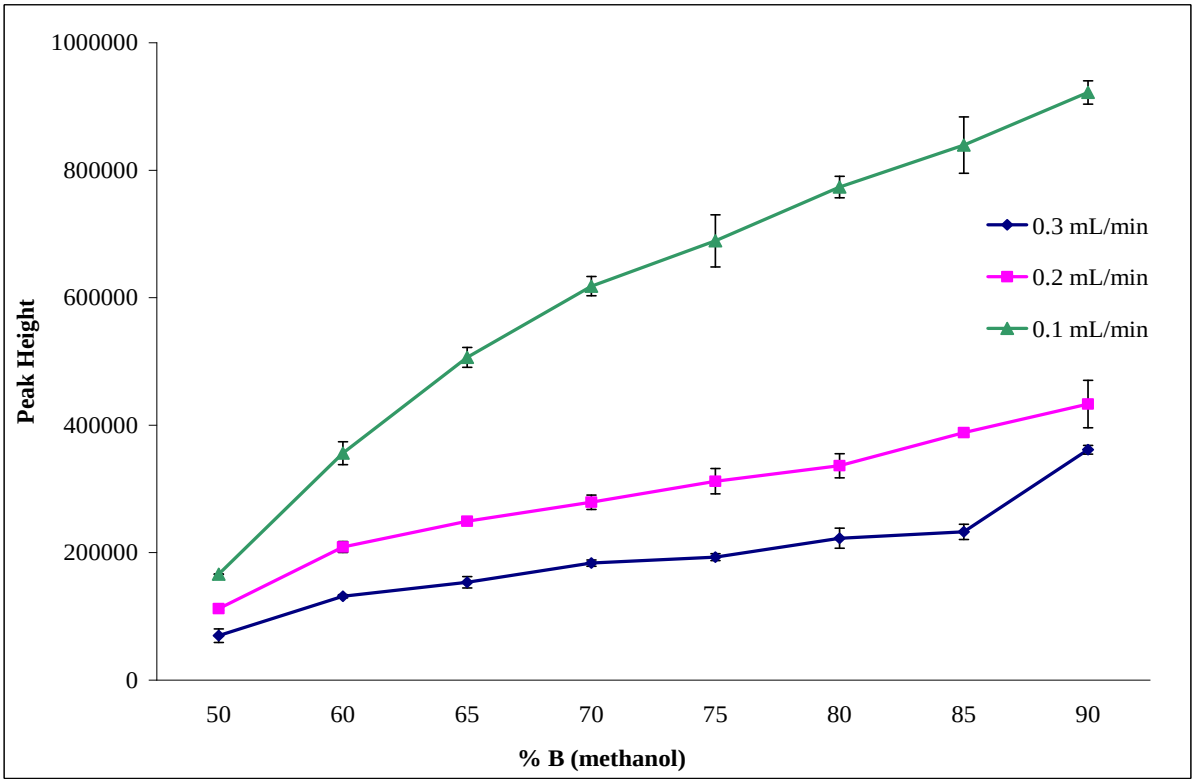


Figure 3.21 Peak height (± SD) (n=4) observed in the mass spectra of cortisol collected at 50-90%v/v MeOH and flow rates 0.1-0.3 mL/min

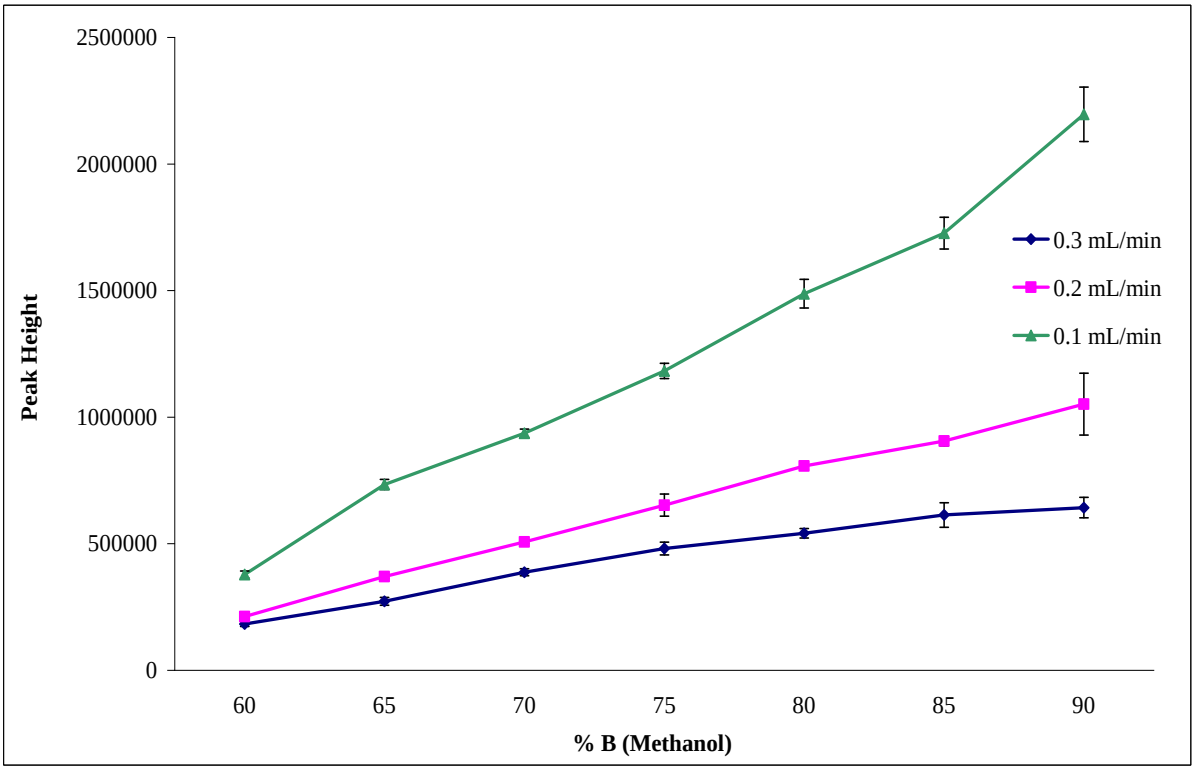


Figure 3.22 Peak height (± SD) (n=4) observed in the mass spectra of testosterone collected at 50-90% v/vMeOH at flow rates 0.1-0.3 mL/min

3.3.7 Ramped gradients

Various ramped gradients were tested to optimise peak height, area and signal to noise ratio (table 3.1 in methods section). All gradients were analysed at a flow rate of 0.3 mL/min for peak area, height and S/N ratio for cortisol and testosterone (tables 3.2 and 3.3) and the optimal gradients for the measurement of cortisol and testosterone were analysed at 0.1 mL/min, given this flow rate is optimal for the column dimensions (table 3.4). The highlighted gradients in table 3.4 represent those chosen for further analysis; the criteria was having a retention under 6.5 minutes for both analytes (to keep run time under 20 minutes, when the flow rate was decreased to 0.1 mL/min) and illustrating high peak area, peak height or S/N (highlighted in bold typeset).

Table 3.2 Mean (± SD) of cortisol and testosterone S/N, peak area and height of various MeOH gradients (n=4)

Gradient	Cortisol			Testosterone		
	S/N	Peak area	Peak height	S/N	Peak area	Peak height
1	41.46 ± 6.49	117542±1196	**176832 ± 2562**	25.68 ± 1.04	199755 ± 2974	276543 ± 6825
2	42.95 ± 2.33	124087±3774	**183017 ± 2799**	40.12 ± 2.20	253648±10684	**355294± 14616**
3	35.43 ± 7.24	114980 1334	166575 ± 2967	31.99 ± 1.63	129002 ± 6663	299787± 13868
4	47.77 ± 4.97	113562 ± 691	164207 ± 3276	37.45 ± 1.74	179646 ± 6393	346429 ±17925
5	58.06 ± 6.27	109893 ± 977	161971 ± 3167	38.78 ± 3.30	195370 ± 1857	351748 ± 7805

Table 3.3 Mean (± SD) of cortisol and testosterone S/N, peak area and height of various MeOH gradients (n=4)

Gradient	Cortisol			Testosterone		
	S/N	Peak area	Peak height	S/N	Peak area	Peak height
6	**68.28 ± 8.67**	30014 ± 271	157370 ± 3623	**85.16 ± 4.92**	63736 ± 1282	535595 ± 16698
7	61.37 ± 6.14	26167± 2273	226139 ± 19626	150.55 ± 24.13	79459 ± 4510	575739 ± 32693
8	49.37 ± 4.60	18683 ± 921	124787 ± 5573	15.59 ± 0.85	45325 ± 1844	248773 ± 9283
9	41.67 ± 6.05	17017 ± 662	129438 ± 3680	29.57 ± 3.13	45796 ± 2119	312382 ± 15015
10	56.39 ± 1.31	106067±1492	201836 ± 4233	25.66 ± 3.64	179822 ±4081	311626 ± 4839

Three optimal gradients (1, 2, and 6) were chosen based on the initial analysis after considering retention time, S/N and peak descriptive; these were tested at 0.1 mL/min (table 3.4; figure 3.21). Choosing the appropriate gradient was based on all parameters; including peak retention with the ideal method able to elicit tall intense peaks for both analytes in < 20 mins.

Table 3.4 Mean (± SD) of cortisol and testosterone S/N, peak area and height of three MeOH gradients at flow rate of 0.1 mL/min (n=4)

Gradient	Cortisol			Testosterone		
	S/N	Peak area	Peak height	S/N	Peak area	Peak height
1	127.97 ± 16.46	268386 ± 20515	849358 ± 55212	154.96 ± 23.08	717864 ± 125037	1673889 ± 330601
2	96.91± 24.25	210606 ± 17515	267410 ± 21740	222.41 ± 61.27	502249 ± 39934	2143123 ± 168164
6	186.7 ± 7.74	281739 ± 32420	844682 ± 133658	336.21 ± 61.98	701557 ± 168046	2131729 ± 261496

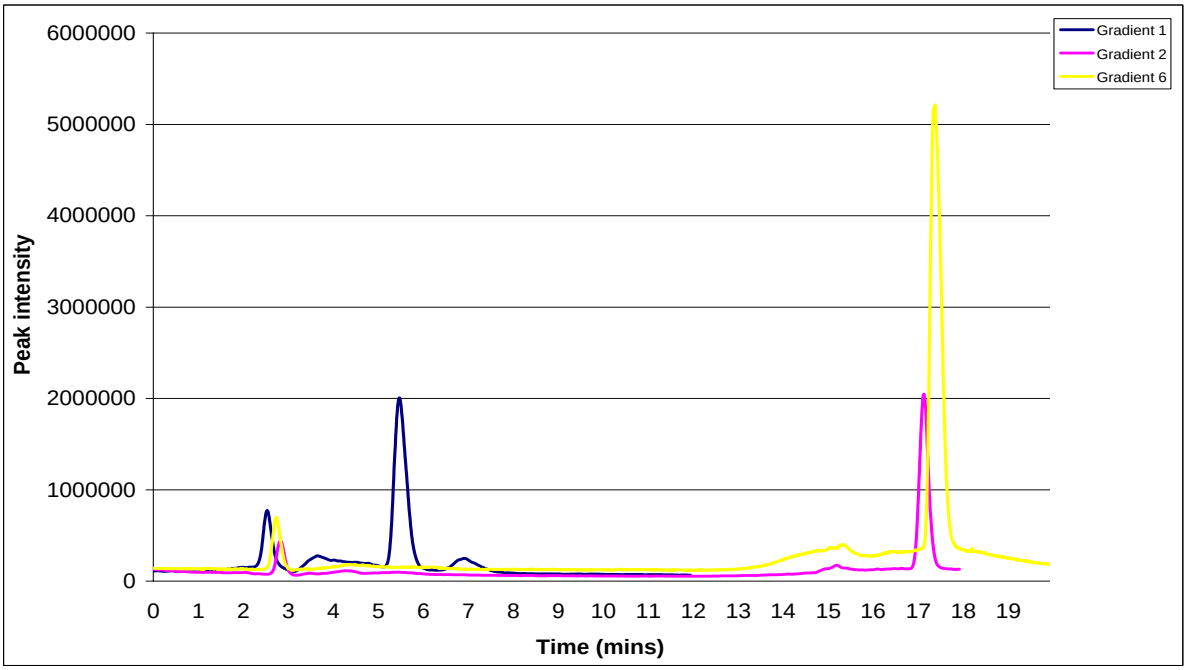


Figure 3.23 Cortisol and testosterone peaks for gradients 1, 2 and 6 at flow rate 0.1 mL/min

Gradient 6 was chosen as it produced a higher S/N ratio for testosterone, compared to the other gradients and S/N ratio for cortisol was comparable to the other gradients. Additionally analyte retention occurred within 20 minutes for both hormones (fig 3.23). S/N ratio is an important parameter in analyte quantification as a ratio of ≥ five is required to determine the lower limit of quantification, (US Department of Health, 2001) therefore, improvements in S/N will have a direct impact on enhancing the quantification of low levels of steroid hormones in saliva.

3.3.8 Column temperature

This work examined column temperatures ranging from 20-50^{o}C for their effect on cortisol and testosterone peak area with a 70% methanol gradient (table 3.5). For cortisol the largest difference in peak area was observed for column temperatures between 20^{o}C and 50^{o}C with 25^{o}C higher by 6.9%. For testosterone the largest difference in peak area was between column temperatures of 25^{o}C and 40^{o}C and 40^{o}C was higher by 4.2%. Given the potential for intra assay precision to be on average 7.0% for cortisol and testosterone (Jonsson *et al.*, 2003; Matsui *et al.*, 2009), it is unlikely that these values represent a true increase. A column temperature of 25^{o}C was chosen to maintain consistency and minimise the proposed negative effects of high temperature on column lifetime (Vanhoenaker and Sandra, 2006). Column temperature has been shown to have a strong influence on peak retention and broadening, thus potentially improving selectivity and S/N ratio (Bowermater and McNair, 1984; Vanhoenaker and Sandra, 2006) however no differences were identified in this study. The chosen temperature produced sufficient peak separation and minimal peak broadening.

Table 3.5 Peak area (±SD) for cortisol and testosterone with column temperatures ranging from 20-50 ^{o}C (n=3)

Column temperature (^{o}C)	Cortisol peak area	Testosterone peak area
20	64346 ± 473	168555 ± 433
25	61546 ± 160	166584 ± 412
30	61503 ± 41	170098 ± 5069
40	62946 ± 2357	172810 ± 1631
50	60404 ± 1740	167323 ± 2998

3.3.9 pH

Mobile phase pH can impact on retention consistency and ionisation efficiency. pH varies with the concentration of solvent and also amount of acid modifier such as formic acid. Samples were analysed with the three percentages of formic acid. Results revealed that 0.1% formic acid produced higher cortisol peak area than 0.2% (15.8 %) and 0.5% formic acid (25.1 %). For testosterone 0.1% formic acid produced higher peak area than 0.2% (9.5 %) and 0.5% (9.8%) (figure 3.24). Retention times were consistent at 0.1% formic acid which suggests sufficient buffering.

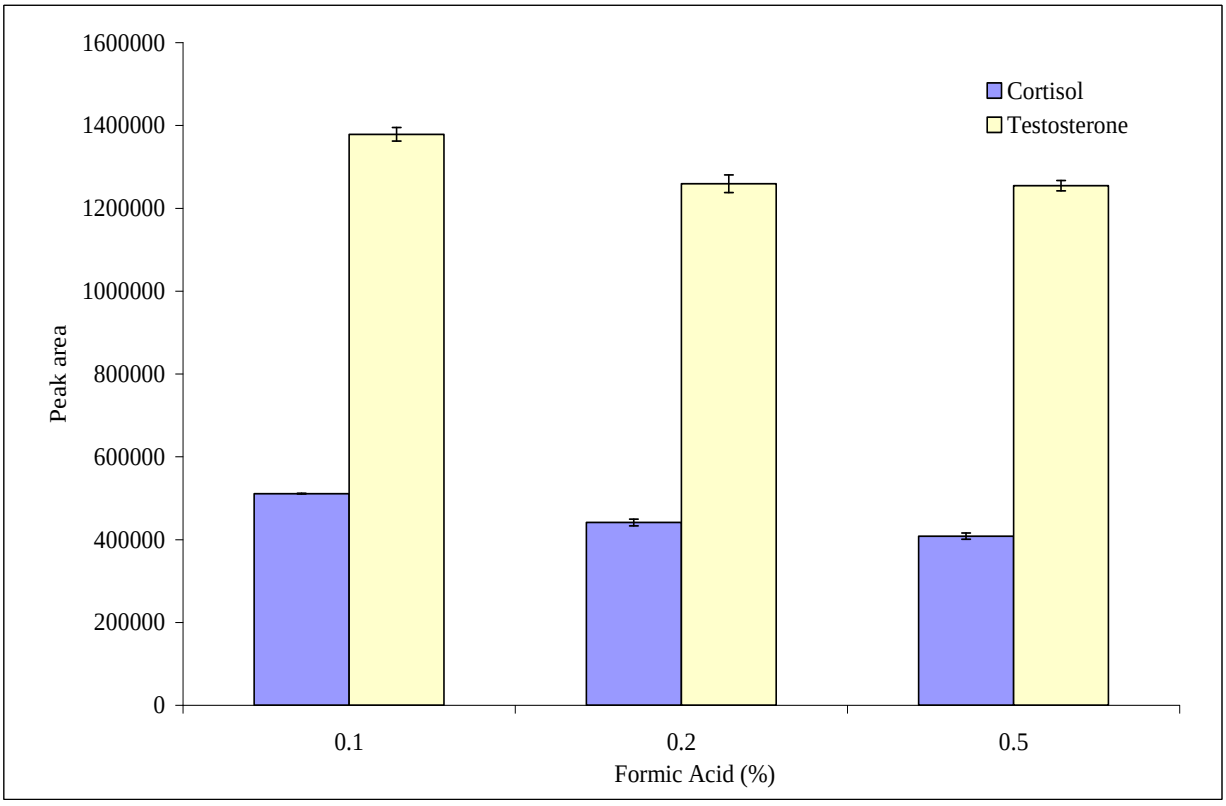


Figure 3.24 Peak area (±SD) (n=4) observed in the mass spectra of cortisol and testosterone (50 ng/mL stock) collected with an isocratic gradient of 70%v/v MeOH containing 0.1, 0.2 or 0.5 % formic acid

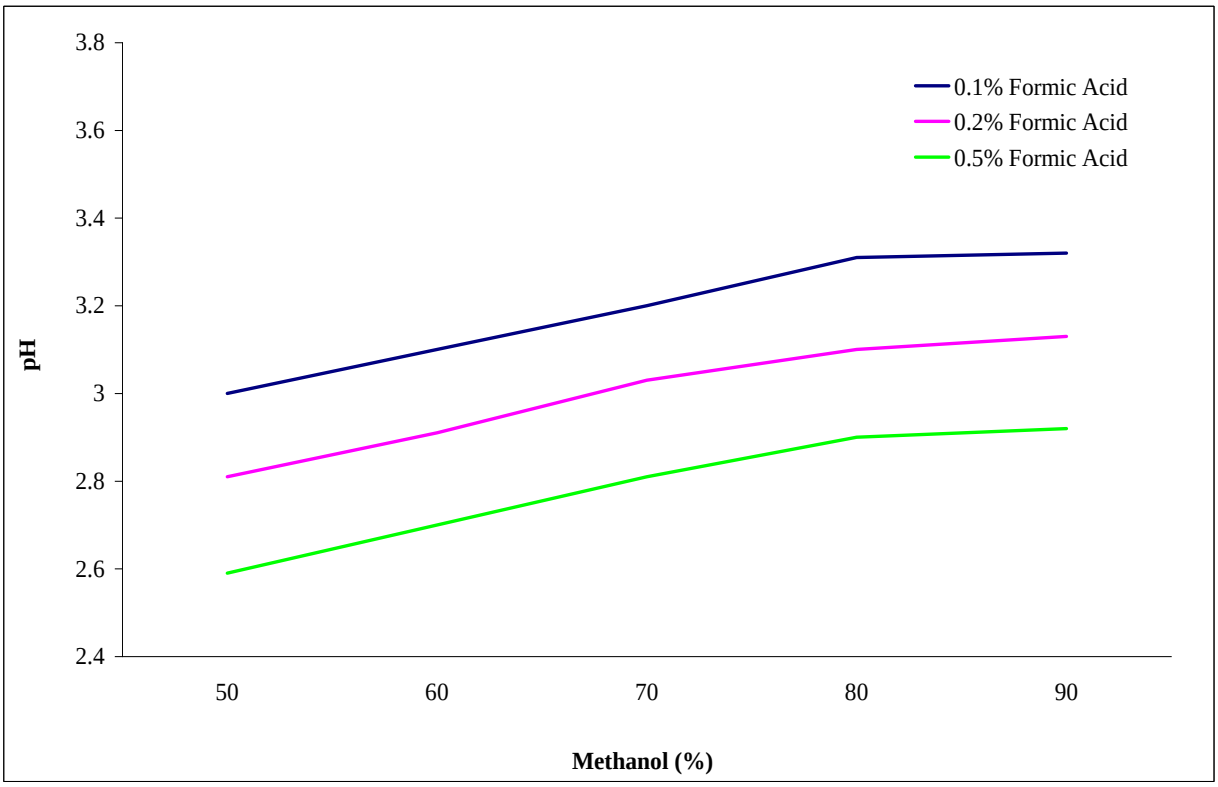


Figure 3.25 pH of 50-90% methanol with 0.1, 0.2 or 0.5% formic acid

Higher peak area with a mobile phase containing 0.1% formic acid is likely to be due to a combination of factors. Firstly, a low pH can contribute to ion suppression on the column, this reduced ionisation of column silanols which can interfere with peaks causing peak tailing (Alexander and Dolan, 2009). A low pH is known to improve ionisation during ESI, cortisol and testosterone have an acid dissociation constant (PkA) of >10 therefore at a low pH the analytes should be completely ionised. However, using high amounts of buffer can cause peaks to co-elute as they are not retained on the column, it is also thought to supress ionisation through high numbers of ions and droplet saturation which prevents ionisation of target analytes (Benijits *et al.*, 2004). Formic acid has a PkA of 3.8 therefore it is advised it can buffer a pH of 2.8 to 4.8. Analysis of pH at of a range of solvent concentrations (50-90% MeOH) with 0.1% and 0.2% and 0.5% formic acid revealed a linear increase in pH as methanol concentration increased (figure 3.25). At 0.2% FA (<55% MeOH) and 0.5% formic acid (<60% MeOH) pH has exceeded the buffering range of formic acid and therefore pH stability cannot be guaranteed (LaserChrom, 2011). In conclusion, 0.1% formic acid is acceptable for buffering methanol concentrations in the mobile phase employed in this method.

3.3.10 Further LC-MS optimisation

To improve the sensitivity of the LC-MS method and lower the detection limit of steroid hormones, several options exist including derivatisation to change the chemical structure of a compound and additionally the use of tandem mass spectrometry, to detect fragmented ions to reduce background interference (Jiao *et al.*, 2002).

Hydroxylamine derivatisation

Hydroxylamine derivatisation was conducted with testosterone based on the method by Kushnir *et al.*, (2006). An initial stock of 1000 ng/mL revealed a resolved peak of *m/z* 304, which indicated testosterone was successfully derivatised (figure 3.26B) and comparison of the peak areas suggests a four fold improvement with derivatisation. However, a co-eluting compound of *m/z* 304 was visible when derivatising low levels of testosterone (10 ng/mL; figure 3.27). Various experiments were conducted to investigate the cause of this interference with SPE, vacuum drying and column contamination excluded.

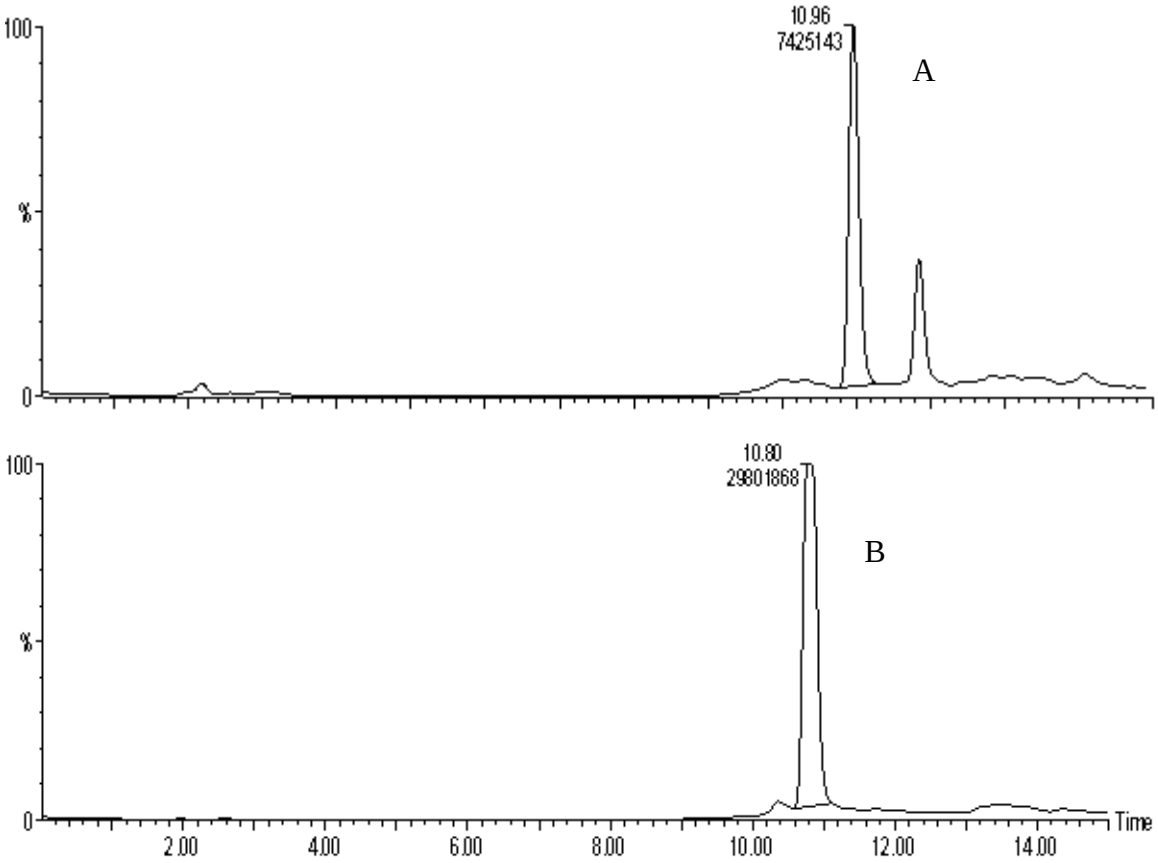


Figure 3.26 Underivatised, m/z 289 (A) and derivatised (m/z 304) testosterone stock (B) (1000 ng/mL)

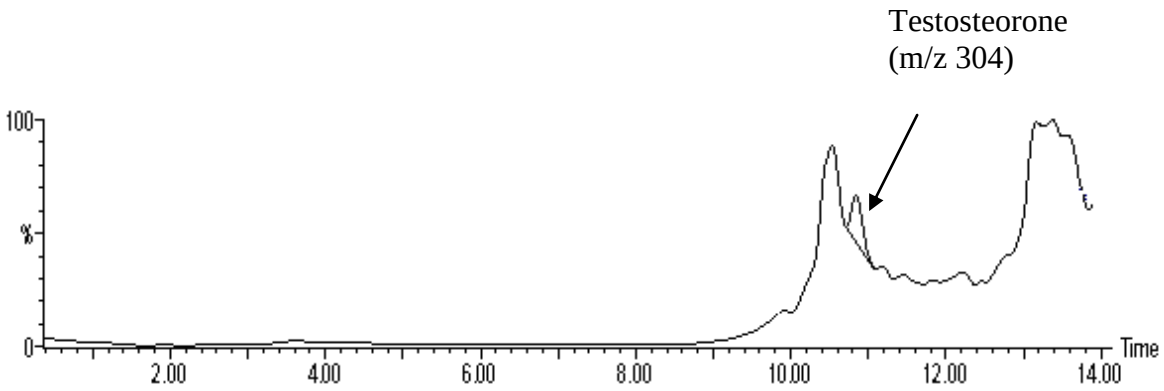
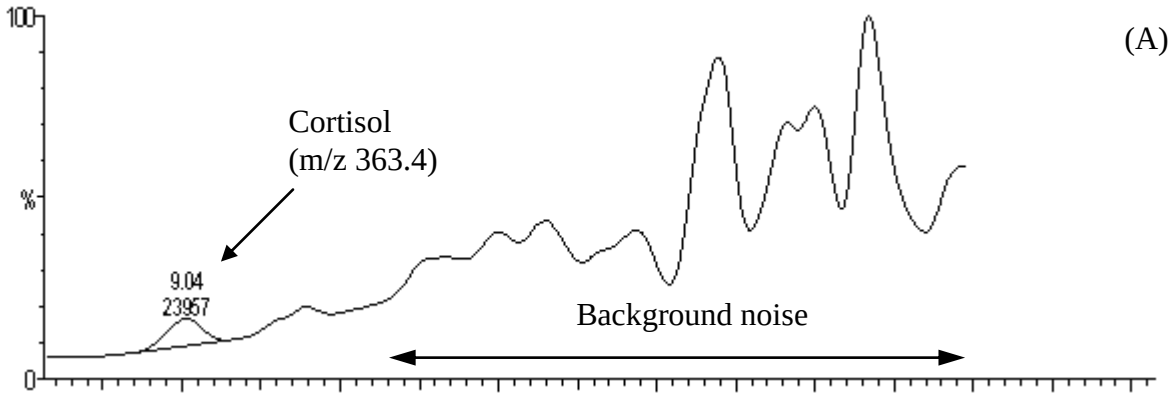


Figure 3.27 Derivatised testosterone (m/z 304) (10 ng/mL)

In conclusion, due to the co-eluting peak at m/z 304 it was very difficult to quantify derivatised testosterone within the reference range for men, however further work by our research group indicates a three fold improvement in testosterone quantification with hydroxylamine derivatisation (Nielsen *et al.,* 2011; unpublished data) This improvement is less than that proposed by Kushnir *et al.,* (2006) who reported testosterone detection could be improved 5-10 fold with this method. However, derivatisation lead to a compromise in cortisol quantification levels; (figure 3.28) therefore, alternative ways to improve quantification of testosterone should be investigated. There are other methods which have been used to derivatise testosterone such as with HMP (Higahsi *et al.,* 2005; Shibayama *et al.,* 2009); pyridine (Licea-Perez *et al.,* 2008) and 2, 6-diaminopyridine (DAP) (Yamashita *et al.,* 2009) and investigation of these methods could be undertaken to establish if improvements in quantification by derivatisation are possible without compromising detection of cortisol. Derivatising compounds has been shown to improve ionisation and decrease LOD by increasing volatility and the change in structure has the potential to improve proton affinity.



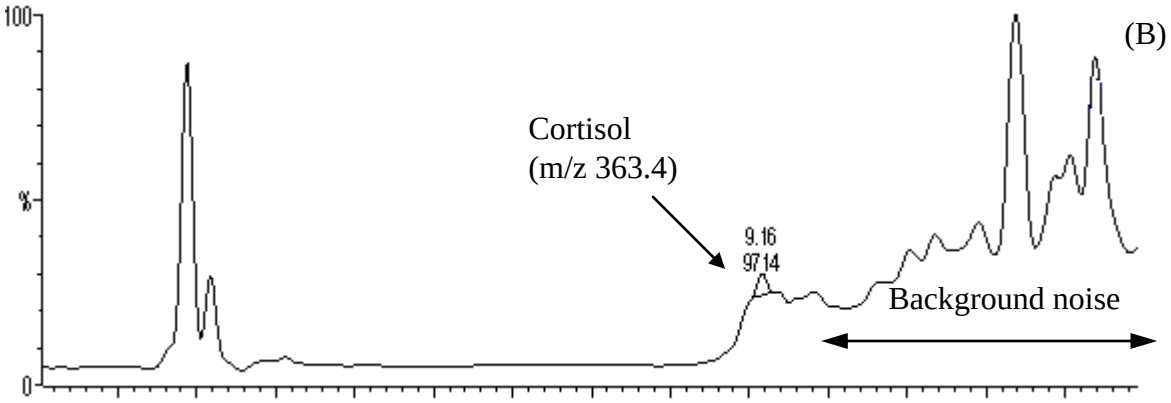


Figure 3.28 Cortisol in an underivatised saliva samples (A) and derivatised (B)

Recent results from our group support reports of improved detection of testosterone with hydroxylamine derivatisation in the literature. There is evidence that co-eulting peaks may be able to be moved chromatographically or through fragmentation by employing MS/MS (Kushnir *et al.,* 2006; Kalhorn *et al.,* 2007).

Tandem Mass Spectrometry

Another method to improve sensitivity in LC-MS may be to employ tandem mass spectrometry (LC-MS/MS). LC-MS/MS can be used to effectively suppress background noise from the sample matrix and greatly enhance sensitivity if the instrument is properly tuned and operated (Jiao *et al.,* 2002). Tandem mass spectrometry has been used to quantify salivary cortisol and testosterone in the reference range for saliva (Matsui *et al.,* 2009; Kusukake *et al.,* 2009). However, similar LOQ have been achieved for quantification of salivary cortisol with LC-MS (Katoaka *et al.,* 2007) and this suggests that LC-MS/MS may not be required to quantify cortisol and testosterone in salivary samples. Although, the specifications of the mass spectrometer used in the latter study are not specified therefore direct comparison of the methods is difficult.

3.4 Summary

The first objective of this research project was to develop a method to measure salivary cortisol and testosterone with LC-MS. This chapter encompassed optimisation of LC and MS parameters for this method with MS parameters manipulated first. Ion selection was based on a full scan across the mass spectra and this identified protonated ions as the dominant ions. Optimisation proceeded using a single ion scan for the protonated form of cortisol and testosterone (m/z 363.4 and m/z 289.4 respectively). Protonated ions of

deuterated forms of cortisol (m/z 365.4) and testosterone (m/z 292.4) were also identified as most abundant. Cone voltage appears to be the most influential MS parameter on peak area of all the MS parameters tested. Cone voltage has a two pronged effect on ions as firstly, when optimised it attracts a high number of ions into the ion block and this has a direct effect on signal strength and detection limits. In this work this equated to an improvement in cortisol and testosterone peak area of over 130%. Secondly, high cone voltage is linked to insource fragmentation; therefore if this parameter is set incorrectly high ion energy can lead to fragmentation and ultimately a reduction in analyte ions reaching the detector. Optimisation of capillary voltage suggests this parameter does affect peak area of cortisol and testosterone. However, less dramatic changes were observed than for cone voltage. Similar conclusions regarding influence on peak area were drawn for low and high mass resolution and the carbon 13 peak was identified to ensure peak resolution.

LC parameters were also optimised for detection of cortisol and testosterone. Manipulation of solvent type allowed for assessment of this parameter on peak area and analyte retention, in this method MeOH produced higher peak area for both analytes of over 25%. Additionally solvent flow rate had a considerable effect on peak area and peak height with a slower flow rate producing the best results. The latter is likely to be linked to the Van Deemter peak broadening theory (Van Deemter, 1956) which implied that optimal flow rate will give a higher number of theoretical plates in the column and lead to higher column efficiency and narrower peaks. The lower flow rate is also likely to improve ion transmission, as smaller electrospray droplets lower the threshold of the Rayleigh limit and provide more efficient transfer from the liquid to gas phase (Gale and Smith, 1993). Combined, these translate to a higher percentage of ionisation; therefore more ions reach the detector. Mobile phase pH is a further parameter which has large influences on ionisation and it is important to add an optimal level of acid buffer to maintain retention and ionisation efficiency. 0.1% formic acid is commonly used in LC and this work supports using this percentage of acid. Dissimilarly, column temperature had negligible impact of peak area and therefore a temperature of 25^{o}C (slightly above room temperature) was employed to minimise temperature effects on retention time.

Finally, considering the low levels of testosterone in saliva, work was conducted to investigate ways to improve ionisation of this compound. In conclusion, derivatisation

may offer improvements in detection of testosterone with reaction of hydroxylamine to produce a testosterone oxime derivative. However, further investigation is required to confirm the true improvements gained with this technique and assess the impact for cortisol detection. This could be achieved through optimisation of the derivatisation method and analysis with LC-MS/MS to reduce background interference.

4. OPTIMISATION OF SAMPLE PREPARATION

With its ease of collection and established correlations with blood (Vittek *et al.,* 1985; Kirschbaum and Hellhammer, 1994) saliva has become a widely used medium to monitor cortisol and testosterone. However, there are two main issues when attempting to quantify hormones in saliva with LC-MS. Firstly, the saliva matrix, can cause ion suppression, and high noise levels subsequently produce poor ion detection and S/N ratio through the 'matrix effect'. This effect is also observed in other biological matrices such as blood and urine. Secondly, concentration of cortisol and testosterone are lower in saliva than blood (10 fold) and this creates a challenge to accurately quantify them. Importance is placed on cleaning the sample prior to analysis employing a method that produces high analyte recovery. Established techniques such as protein precipitation and solid phase extraction (SPE) are commonly used to reduce the effect of matrix noise and to concentrate the sample for analysis with mass spectrometry. This chapter includes examination of saliva sampling considerations and matrix effects, optimisation of an SPE extraction method for cleaning saliva prior to LC-MS analysis and finally investigation of sample recovery.

4.1 Saliva

4.1.1 Saliva composition

Saliva is a clear, slightly acidic mucoserous endocrine secretion. It is composed of more than 99% water with a normal pH of 6-7. Saliva contains a variety of electrolytes including sodium, potassium, calcium, magnesium, bicarbonate and phosphates as well as immunoglobulins, proteins, enzymes, mucins, urea and ammonia (Humphrey and Williamson, 2001) (figure 4.01) Secretion of saliva is controlled by a salivary centre composed of nuclei in the medulla (Grant *et al.,* 1988) and is triggered by mechanical, gustatory and olfactory stimuli (Humphrey and Williamson, 2001). Additionally many small biomarkers including hormones are present in saliva. Most steroids including cortisol and testosterone enter the saliva through diffusing through the cells of the salivary glands and their concentration is not dependent on flow rate (Vining *et al.,* 1983).

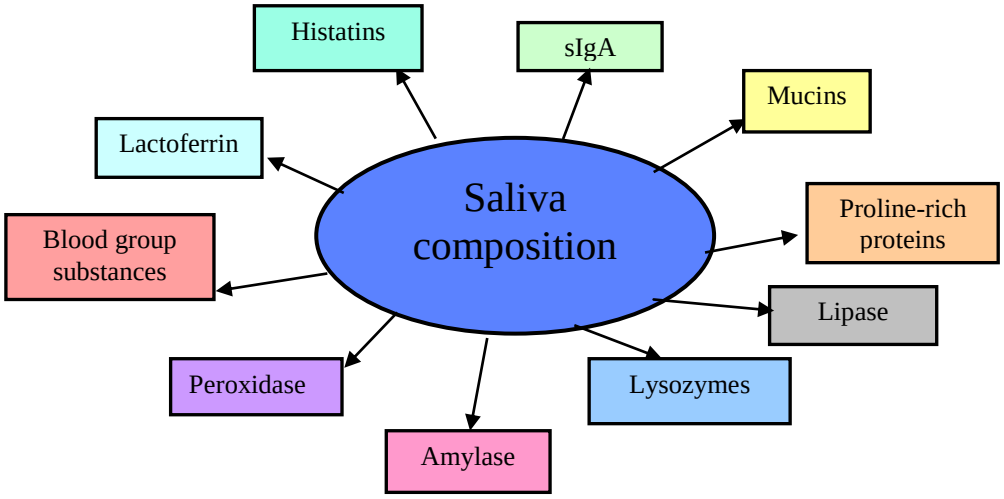


Figure 4.01 Components of saliva

4.1.2 Salivary measures

Salivary measurement has many benefits over blood measures. Sample collection is less invasive therefore less likely to induce a stress response (Kraemer *et al.*, 2001), also the sample can be collected by patients or study participants and there is no need for medical personnel or researchers to be present. This is particularly advantageous in large cohort studies and studies in children (Jessop and Turner-Cobb, 2008). Additionally, salivary measures are often indicative of the free or 'biologically active' biomarkers, as they diffuse from the blood into the mouth and are not bound by albumin therefore may indicate the levels which initiate the body's stress response (Humphrey and Williamson, 2001). However representing free hormone levels means low concentrations of cortisol and testosterone compared to blood (table 1.1); and this can lead to problems with accurate detection in samples from women and children (Kushnir *et al.*, 2006).

4.1.3 Collection of saliva

Various studies assess appropriate collection methods for salivary hormones. Poll *et al.*, (2007) showed a higher correlation between salivary and serum cortisol levels when collecting with cotton Salivettes rather than passive drool. However, cotton Salivettes have been shown to elicit low hormone recoveries of 88% for cortisol and 62% for testosterone (Groschl and Rauh, 2006), therefore use in steroid analysis is discouraged. With regards stimulating saliva production, Dabbs *et al.*, (1991) and Booth *et al.*, (2003) investigated the

effect of chewing gum on salivary testosterone and found that levels tended to increase within the first minute of chewing, but were not significantly above baseline at any other time point. Participants should therefore be advised to chew for at least a minute before providing the saliva sample. Additionally, the purpose of hormone measurement should be considered with collection. Resting cortisol and testosterone levels exhibit a diurnal variation (Baxendale *et al.,* 1980), therefore timing of sampling should be considered if collecting during the awakening response. After this period levels are fairly stable and high volumes can be sampled. When measuring the acute effects of a stressor short sample collection times are required to capture the immediate hormonal response to the stressor. When stimulated by chewing or by moderate strengths of citric acid, whole saliva flow rate increases from the resting value of around 0.3–0.65 mL/min (Bertram, 1967) to around 1.5–6.0 mL/min (Heintze *et al.,* 1983) and there is large individual variation. Additionally, dehydration through sweat loss during exercise may decrease saliva flow rate (Ford *et al.,* 1997) and must be considered. Therefore, when collecting saliva samples to assess the acute stress response during and after exercise; volume will be restricted by the sampling time. Volume may also be affected by vasoconstriction of the arterioles surrounding the salivary glands resulting in lower salivary volume (Chicharro *et al*., 1998), which has been postulated during and after intense exercise. Therefore, during a stimulated sample over a three minute period, it is realistic to collect between 2 mL and 3 mL. Concentration of samples prior to MS analysis can improve detection limits however sample volume restrictions during and after exercise limits the factor of concentration.

4.1.4 Storage of saliva

Correct storage of saliva is important to minimise sample degradation and reductions in hormone concentration which can limit detection. Granger *et al.,* (2004) studied the stability of salivary testosterone and found when stored at -20^{o}C there was an 18% decline in testosterone after six months but at -80^{o}C there was no significant change after 36 months. This is supported by Whembolua *et al.,* (2006) who found higher testosterone in samples stored at -80^{o}C on the day of collection compared to samples stored at room temperature. Examining storage at higher temperatures, Granger *et al.,* (2004) reported a significant increase in testosterone levels after storage at 4^{o}C for four weeks and this was suggested to be due to bacterial growth interfering with the ELISA assay used in this study. Although, conversely, Whembolua *et al.,* (2006) reported that after 10 days at room temperature salivary testosterone declined 39% and there was no correlation between

bacteria colony counts and salivary levels of testosterone. Cortisol appears to be more stable than testosterone, supported by findings from Aardal and Holm (1995) who revealed salivary cortisol was stable at room temperature for at least 7 days and at -20^{o}C for nine months. Similarly, Kirschbaum and Hellhammer (2000) suggest that salivary cortisol can be stable at room temperature for up to four weeks and however for longer storage temperatures of -20^{o}C or lower are recommended. These results support storage of saliva samples at -80^{o}C prior to measuring cortisol and testosterone.

4.1.5 Matrix effects and ion suppression

Despite the benefits of salivary measurement, like other biological fluids it can affect ionisation during ESI through 'matrix effects'. Matrix effects can be defined as the alternation of ionisation efficiency in the presence of co-eluting substances and this concept was first described by Tang and Kebarle (1993). Various methods are used to measure ion suppression, commonly this is achieved through post extraction addition where a matrix sample is spiked with analyte post extraction and the signal of analyte compared with a pure solution with identical concentration of analyte. The matrix effect is calculated by the difference in analyte signal between the samples and is usually presented as a percentage (Taylor, 2005).

There have been various mechanisms proposed for the cause of matrix ionisation suppression during ESI. King *et al.,* (2000) have shown that matrix effects are the result of competition between non volatile matrix components (such as salts) and analyte ions for access to the droplet surface and transfer to gas phase. Any mechanism that might decrease the production rate of small droplets and ultimately gas phase ions could participate in ionisation suppression. High concentrations of non voltalite materials present in the spray with the analyte increase attractive forces keeping the droplets together. Therefore, ions in the unevaporated portion of the electrospray liquid may collect on the interface plate of the mass spectrometer and fail to enter the detector. Analyte polarity has been shown to potentially cause ion suppression of similar sized analytes: in mixtures, the more non-polar analytes have a higher affinity for the ESI droplet-air interface where the effective competition for the excess charge results in higher signal intensities in the mass spectrum (Abaye *et al.,* 2011a; 2011b). Another mechanism involves the mass and charge of individual analytes, where molecules of higher mass can suppress the ionisation of smaller molecules (Sterner *et al.,* 2000), however the presence and the cause of signal

suppression can be difficult to determine. Ionisation suppression can affect the development of MS methods as when ion suppression occurs the sensitivity and LOQ may be adversely affected (Buhrman, 1996). For both qualitative and quantitative LC-MS understanding and eliminating this cause of signal suppression is essential. The impact of ion suppression can be reduced through chromatographic separation so the analyte elutes away from interfering compounds or alternatively modification of the sample extraction method (Avery, 2003). Increasing retention of analytes to avoid elution in the solvent front where the majority of matrix effects occur is one method to reduce the effect of co-eluting compounds (Muller *et al.*, 2002). Furthermore, addition of an internal standard, which co-elutes with the compound of interest can control for matrix effects as they would both experience the same interference from ion suppression (Bonfiglio *et al.*, 1999; Annesley, 2003).

Finally, cleaning the sample prior to analysis can also be beneficial. SPE and liquid-liquid extraction have been shown to reduce ion suppression through removal of interfering compounds that can compete for charges during ESI or interferes with desolvation (Bonfiglio *et al.*, 1999; Muller *et al.*, 2002).

4.1.6 Sample cleaning

The importance of sample cleaning to reduce ionisation suppression has been illustrated as electrolytes and other components in a saliva matrix can potentially interfere with ionisation of the analytes of interests and directly influence detection limits. Additionally sample concentration and recovery are important parameters to optimise for improvements in detection and quantification in saliva.

Solid phase extraction

SPE is a widely used sample-preparation technique for the isolation of selected analytes, usually from a mobile phase (gas, fluid or liquid). Analytes are transferred to the solid phase where they are retained while larger compounds are removed (washing) and finally the analytes of interest are eluted. The principal goals of SPE are:

- matrix simplication (sample clean-up)
- trace enrichment (concentration)
- medium exchange (transfer from the sample matrix to a different solvent or to the gas phase) (Poole, 2003)

SPE has been widely used in the preparation of serum, plasma and saliva prior to analysis for cortisol (Kataoka *et al.,* 2007; De Palo *et al.,* 2009) and testosterone (Chang *et al.,* 2003; Shibayama *et al.,* 2008; Yamashita *et al.,* 2009).

4.1.7 Recovery of analytes

The chosen cleaning techniques can affect recovery of analytes and this is especially important for testosterone as losses exacerbate the difficultly in detection of very low levels in saliva. Chang *et al.,* (2003) reported testosterone recoveries of 91% at 2 ng/mL and 10 ng/mL and recovery of 96% was found by Cawood *et al.,* (2005). Similar results were also shown for cortisol by AbuRuz *et al.,* (2003) (90%) and Kataoka *et al.,* (2007) (95%).

Considerations for high recovery post SPE include the method used to drying the analyte eluent. This is important in terms of productivity, reliability and recovery of the analyte. Three methods of drying (freeze drying, vacuum centrifuge and nitrogen) have been examined for quantification of salivary cortisol levels by Nelson *et al.,* (2008). They concluded that evaporation of samples under a nitrogen flow is time consuming has more room for human error. Although they reported no difference in recovery of cortisol between the drying methods benefits of using freeze drying or vacuum centrifuge for drying the eluent included improved efficiency and reproducibility.

4.2 Methods

4.2.1 Calibration standards

Calibration standards (4mL) were prepared in water as described previously in Chapter 2. Concentrations of 0, 1, 2, 5 and 10 ng/mL of cortisol and 0, 0.1, 0.2, 0.5 and 1.0 ng/mL of testosterone and IS (3 ng/mL and 0.3 ng/mL deuterated cortisol (d_2) and testosterone (d_3) respectively (from QMX laboratories, Thaxted, UK)) were added to all calibration standards. Prior to separate analysis of single analytes, standards were prepared containing only cortisol and cortisol (d_2) IS or only testosterone and testosterone (d_3) IS, the latter was also prepared in the higher range of 0-10 ng/mL (Chapter 2; table 2.2). 1000 µL of each stock was used for analysis. Known concentration samples (1000 µL) were also prepared (1.89, 4.72 and 7.54 ng/mL cortisol and 0.19, 0.47 and 0.75 ng/mL testosterone, IS 3

ng/mL and 0.3 ng/mL respectively (chapter 2). Prior to analysis all samples were diluted with 1000 µL water and underwent solid phase extraction (SPE) after which the eluent was dried and the sample reconstituted in 100 µL (concentrating 10 times).

4.2.2 Matrix effect

Mean LC-MS spectra of spiked analyte free saliva (20 ng cortisol and 20 ng testosterone) was compared with negative controls (water) spiked with the same concentration of both analytes. Analyte-free samples were prepared with activated charcoal treated saliva (Shibayama *et al.*, 2009). 500 µL saliva was pre-treated (1 mL MeCN added, centrifuged 3000 RPM, 5 mins) then SPE was conducted with 200 mg HyperSep C18 cartridge (Thermo Scientific, Bellefonte, PA) the SPE eluent was evaporated (vacuum centrifuge, 500 x G), reconstituted (500 µL MeOH: water (60:40)) and spiked with 20 ng/mL cortisol and testosterone. Positive control samples of 500 µL MeOH: water (60:40) were spiked with cortisol and testosterone (20 ng/mL). Two saliva and two control samples were prepared and were analysed in triplicate with LC-MS (70% MeOH isocratic mobile phase, 0.3 mL/min).

4.2.3 Solid phase extraction

The SPE process conducted in these experiments (figure 4.02) involved the following steps. 3mL 200 mg HyperSep C18 cartridges (Thermo Scientific, Bellefonte, PA) were attached to a 12-position vacuum manifold (Phenomenex, UK). Cartridges were conditioned with MeOH (2 mL) followed by water (2 mL) then loaded with the sample (1mL diluted with 1mL water) (total volume 2 mL). A vacuum pressure (< 20 mmHg) was applied to the cartridges for binding the analytes to the cartridges with the correct sample flow rate indicated by individual droplets. Two washes, water (2mL) and MeOH (2mL, 10%) were executed before the analytes were eluted with MeOH (2 x 500 µL). Eluent was evaporated to dryness in a vacuum centrifuge at 45^{o}C and reconstituted in 100 µL mobile phase (50:50 MeOH: water).

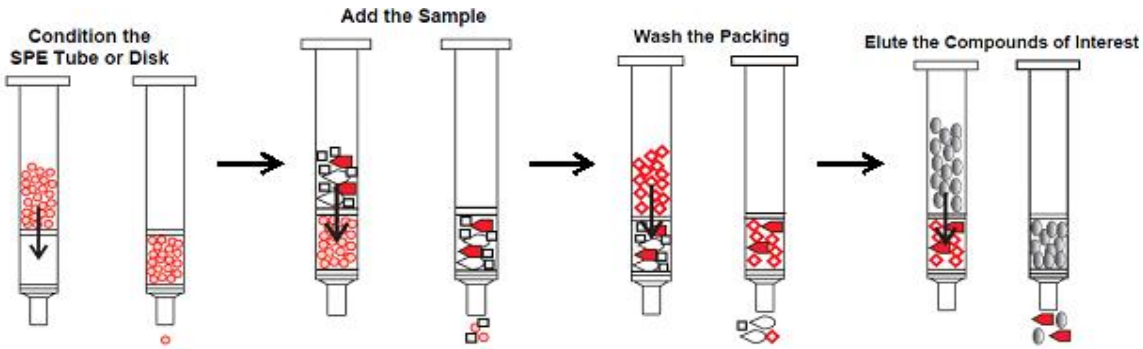


Figure 4.02 SPE procedure (Supelco, 1998; Buletin 910, Introduction to Solid Phase extraction)

Washing during SPE was assessed (10, 40 and 50% MeOH) for its effect on sample recovery. Cortisol and testosterone stock 50 ng/mL was compared with samples which had undergone SPE with a 10, 40 and 50% MeOH wash. Three samples were analysed for each wash percentage and were analysed in duplicate. Three samples (50 ng/mL) were prepared without SPE and repeated in duplicate as controls. Peak areas of cortisol and testosterone were recorded for all samples and recovery calculated using the equation details in chapter 2.

To analyse the effect of concentrating samples on accuracy, calibration samples were prepared with 0.1, 0.2, 0.5 and 1.0 ng/mL of testosterone and 1.0, 2.0, 5.0 and 10 ng/mL cortisol with 0.3 ng/mL deuterated testosterone and 3 ng/mL deuterated cortisol respectively (table 2.2). Samples underwent SPE and were concentrated 10, 20 or 50 times (table 2.4). Known amounts of cortisol (2, 7 and 8 ng/mL) and testosterone (0.2, 0.3 and 0.8 ng/mL) were also prepared and analysed for accuracy at the same concentrations.

For assessment of sample reconstitution 1 mL samples of 5 and 10 ng/mL (3 ng/mL IS) for cortisol and 0.5 and 1 ng/mL (0.3 ng/mL IS) underwent solid phase extraction and vacuum drying, and were reconstituted with 100 µL of 50% MeoH or 80% MeOH. Peak areas were recorded for both analyte and IS and mean values ± SD plotted graphically.

Table 4.1 Concentration of calibration and saliva samples

Concentration	Original stock volume (µL)	Reconstitution (µL)	Wash and conditioning volume (µL)
10X	500	50	1000
15X	750	50	2000
20X	1000	50	2000

4.2.4 Analyte recovery

Recovery was assessed by analysing two known amounts of cortisol and testosterone (2 ng/mL and 8 ng/mL for both) in triplicate. These samples underwent preparation with SPE (method above, 4.2.3) and were concentrated 10 times. A sample with the same concentrations spiked post SPE (2 ng/mL and 8 ng/mL) and concentrated 10 times was used as the control. The recovery of the method was calculated as the difference in mean peak area of the known samples compared to the peak area of the control sample. The following equation (equation 2) was used and the final value expressed as a percentage of recovery: [Mean peak area of known sample/peak area control sample*100].

4.2.5 Statistical analysis

Matrix effect were analysed with Students paired t-tests and a P value of <0.05 was accepted as significant.

4.3 Results and discussion

4.3.1 Matrix effects

The saliva matrix effect was assessed by comparing the peak areas of cortisol and testosterone from a spiked, charcoal treated analyte-free saliva sample and a positive control (non matrix) sample prepared in water. Both samples were spiked with 20 ng/mL cortisol and testosterone and analysed by LC-MS following SPE cleanup. A paired t-test revealed there was no significant difference in the peak area of cortisol between the matrix and non-matrix samples however for testosterone the matrix sample peak area was significantly lower than the control by 6.3% ($t=-2.662$, $p=0.024$) (figure 4.03). This suggests that noise from the background of a saliva sample may negatively affect peak area and potentially compromise the ability to quantify this analyte. Competition for the total available charge from matrix components can lead to ion suppression and poor detection

limits (Boyd *et al.,* 2008). Additionally, a decrease in surface tension of electrospray droplets may occur as a result of a high concentration of co-eluting compounds (Mallet, 2004). This could decrease the rate of solvent evaporation and probability that the droplet will reach a sufficiently small size that ion evaporation can occur. In turn more ions would remain in the liquid phase and fail to reach the detector (King, 2000). Ion suppression may have contributed to the decrease in testosterone detection in the matrix sample. It is therefore important to optimise cleaning of samples to reduce potential matrix effects.

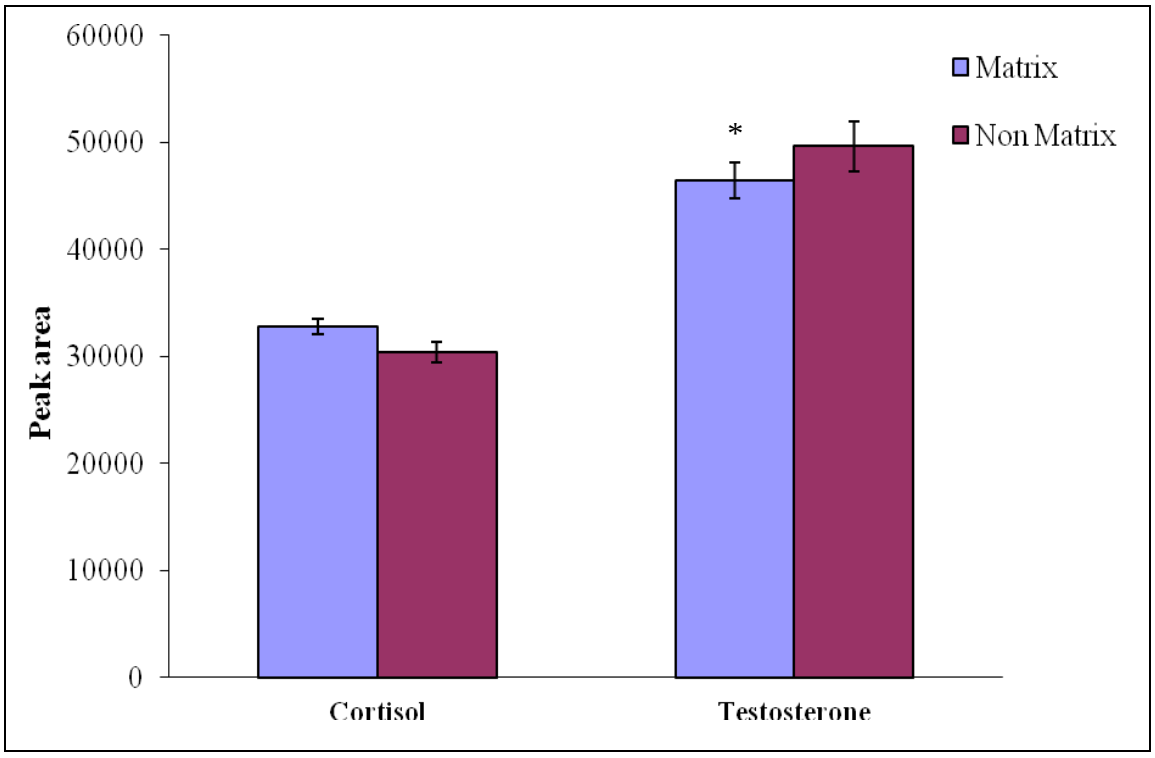


Figure 4.03 Peak area (± SD) of cortisol and testosterone for matrix and non-matrix samples (n=6) *significantly lower than non matrix ($P<0.05$).

4.3.2 Concentration of sample

Samples were concentrated 10, 20 and 50 times for cortisol and testosterone to assess accuracy of quantification of known amounts of each analyte (table 4.2).

Table 4.2 Accuracy (%) for cortisol and testosterone for three control samples (n=3)

Cortisol				Testosterone			
	2 ng/mL	5 ng/mL	8 ng/mL		0.2 ng/mL	0.3 ng/mL	0.8 ng/mL
10X concn	91.5%	92.4%	98.4%	10X concn	80.0%	92.0%	90.0%
20X concn	95.0%	100%	90.8%	20X concn	60.0%	61.2%	66.6%
50X concn	90.5%	97.4%	92.8%	50X concn	63.4%	77.9%	89.5%

The mean accuracy for cortisol at 10, 20 and 50X concentrations were 94.1%, 95.3% and 93.6% respectively, this illustrates that there was no improvement in accuracy with concentration >10X. Similarly for testosterone there is no improvement in accuracy above 10X concentration. Method validation guidelines state that accuracy should be within 20% of the actual value (U.S. Department of Health, 2001) and for concentrations of cortisol meets these criteria. Similarly testosterone meets the criteria above 0.5 ng/mL (concentrating 10 times). The validation guidelines also state that at lower limit of quantification (LLOQ) accuracy should be within 15% and results suggest 0.2 ng/mL is close to this limit. This method is to be applied to samples collected after exercise; therefore volume must be considered prior to concentration as well the capacity of SPE cartridges. It has been established that post exercise a three minute stimulated saliva collection is likely to elicit approximately 3mL saliva; therefore after centrifugation and removal of 500 µL for ELISA analysis, duplicate samples of 1 mL will be stored for LC-MS analysis. With 1:1 dilution prior to SPE the sample is appropriate for 3mL cartridges and reconstitution in 100 µL allows concentration 10 times with the final sample volume sufficient for multiple LC-MS injections.

4.3.3 SPE wash phase

Cortisol recovery decreased as the percentage of organic solvent in the wash phase increased (figure 4.04). A one ANOVA for cortisol revealed 50% MeOH produced significantly lower sample recovery than 10% MeOH (P=0.001) (effect of wash, $F_{(2,17)}$=6.101, p=0.012). No effect of wash was observed for testosterone between 10 % and 50 % MeOH. These findings are in line with those from a study of testosterone

recovery which showed no decrease with 50% MeOH wash in Strata X SPE cartridges (Huq *et al*., 2008). Testosterone is insoluble in water however unlike most steroids cortisol exhibits some solubility and the current findings suggest that a concentration of 50% MeOH may exceed the non polarity of cortisol and elute it from the SPE cartridge. Testosterone is less polar that cortisol therefore requires a higher proportion of organic solvent for elution. In conclusion, a wash phase of 40% methanol or below is acceptable during SPE of these analytes.

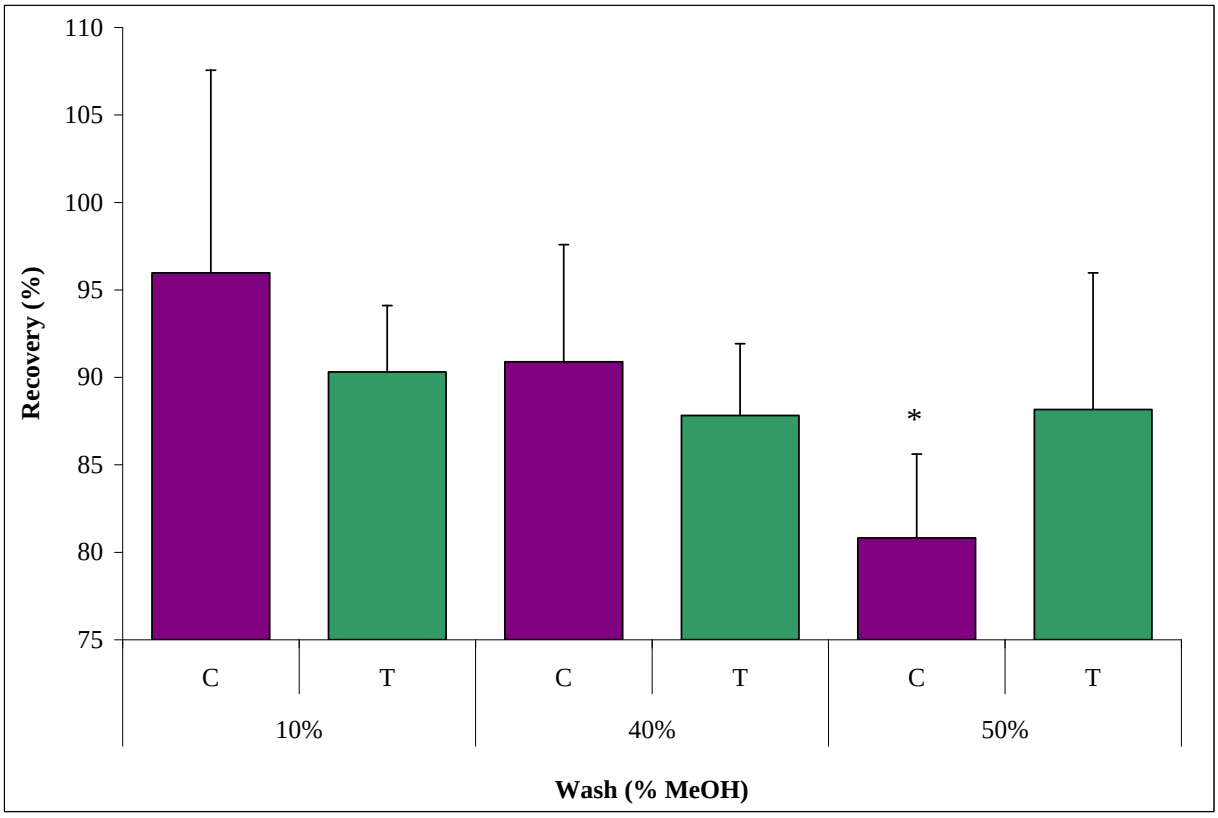


Figure 4.04 Changes in recovery (mean ± SD) for washes of 10, 40 and 50% MeOH (n=6).
*significantly lower than 10% MeOH wash (P<0.05)

In this experiment the error bars were large and machine variations in ionisation may have contributed to some of this difference. The SPE cartridges had a packing volume of 600 mg and guidelines state that the mass of compounds to be extracted should not be more than 5% of the mass of the tube packing (Supleco, 1998). The mass of the packing has a tolerance of 30 mg of compound therefore adding 100 ng would not have exceeded this limit. Conversely an excessive bed weight can result in incomplete elution and therefore this may have affected the results. Polarity of the wash will also impact recovery, a polar solvent such as water will retain the analytes on the stationary phase, and adding a higher

proportion of MeOH will encourage the hydrophobic compound to leave the stationary phase. Less polar compounds such as testosterone will require a higher percentage of organic solvent to dissolve and be removed from the SPE sorbent.

4.3.4 Reconstitution of dried SPE eluent

Results showed that for testosterone (0.5 mg/mL and 1 ng/mL) peak area ratio was higher when reconstituting samples in 80% MeOH compared to 50% MeOH (figure 4.05). There was also a higher peak area ratio for cortisol (5 ng/mL) when redissolving the dried SPE eluent in 80% MeOH compared to 50% MeOH (figure 4.06). These results suggest there is higher testosterone recovery when there is more organic solvent present. Similarly for cortisol (5 ng/mL) PA ratio was significantly higher when reconstituting in 80% MeOH compared to 50% (t=-7.50. p=0.005).

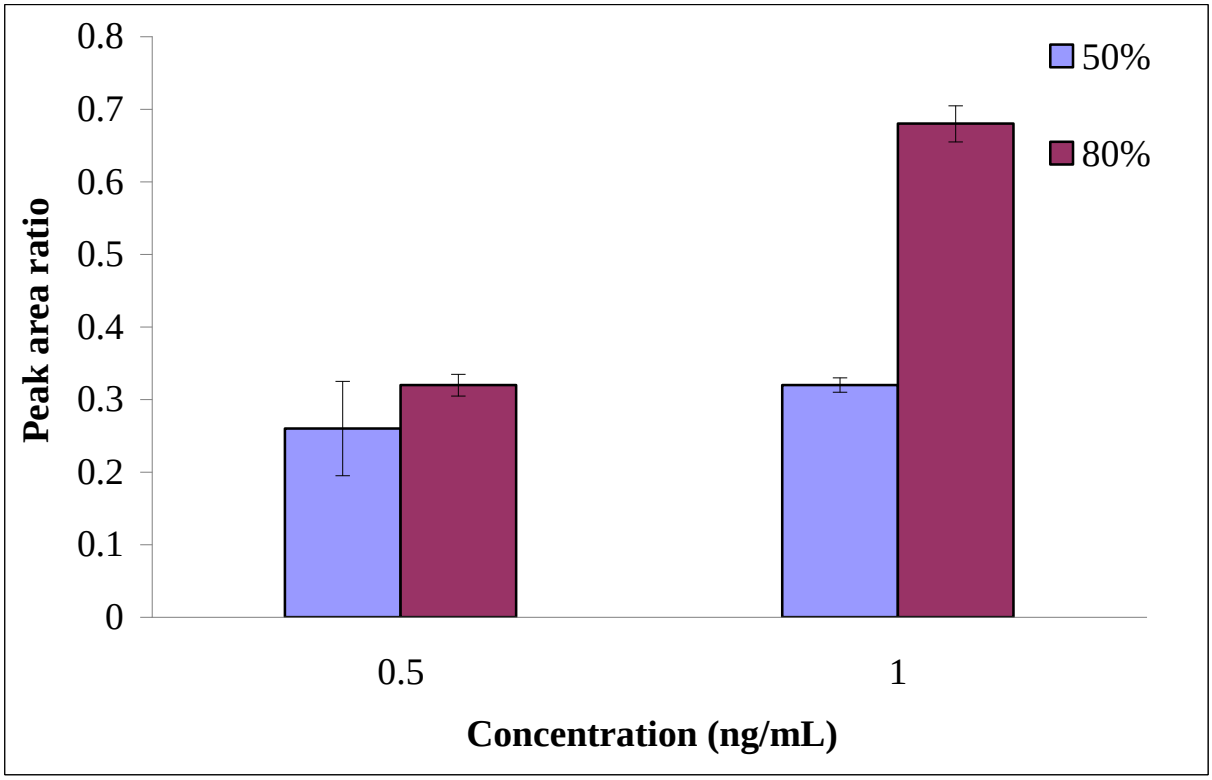


Figure 4.05 Peak area (± SD) of testosterone samples of 0.5 and 1 ng/mL reconstituting in 50% and 80% MeOH (n=4).

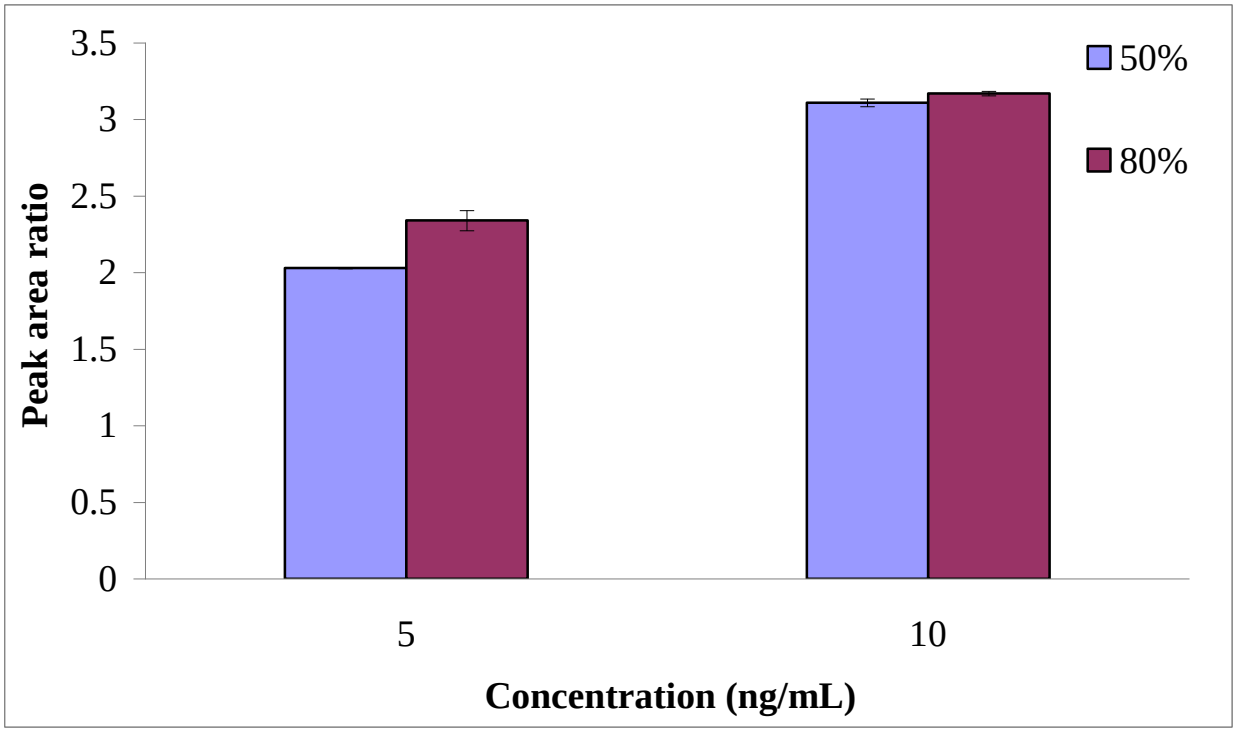


Figure 4.06 Peak area (± SD) of cortisol samples of 5 and 10 ng/mL reconstituting in 50% and 80% MeOH (n=4).

Using peak area ratio controlled differences in ionisation which could be attributed to the sample cleaning and the method error. The findings for testosterone are expected given its requirement for high levels of organic solvent to dissolve. However, cortisol is slightly soluble in water and would be expected to dissolve in 50% MeOH. The findings show that dried analyte should be reconstituted for analysis in 80% MeOH rather than 50%.

4.3.5 Sample recovery

Sample recovery was tested at two concentrations over different days (table 4.3) and results revealed mean recovery was 93.3% for cortisol and 84.8% for testosterone which is in line with other studies measuring these analytes (Chang *et al.,* 2003; Cawood *et al.,* 2005; AbuRuz *et al.,* 2003 and Kataoka *et al.,* 2007); there is no recommended threshold for sample recovery however ideal recovery would be 100% and the extent of recovery of the analyte and internal standard should be consistent (U.S. Department of Health, 2001).

Table 4.3 Mean (± SD) PA of 2ng/mL and 8 ng/mL cortisol and testosterone pre and post SPE (n=6)

Samples	Cortisol		Testosterone	
	2 ng/mL	8 ng/mL	2 ng/mL	8 ng/mL
PA Pre SPE	34881 ± 1173	313934 ±22214	539997 ± 13308	1731725 ± 51892
PA Post SPE	36945 ± 457	289019 ±10834	461220 ±9233	2056264 ± 43707
Recovery (%)	**94.4 ± 4.6%**	**92.1 ± 4.5 %**	**85.4 ± 2.1%**	**84.2 ± 0.6 %**

The reduction in analyte signal reported after SPE is likely to occur from a combination of losses during the SPE process including elution and transfer between vials and the wash phase. The above study used 10% MeOH and previous experiments to compare analyte recovery with different washes suggest this condition is likely to minimise sample losses (figure 4.04). Addition of an internal standard would also control for losses obtained during the SPE process.

4.4 Summary

Saliva samples have been established as a convenient, easy and non invasive alternative to blood measures for measurement of cortisol and testosterone. Collection can be achieved quickly and patients or study participants are able to collect samples themselves if necessary and send them for analysis. Cortisol is stable in saliva for at least one week at room temperature (Aardal and Holm, 1995) and both cortisol and testosterone are stable in saliva for prolonged periods when stored at -80^{o}C prior to analysis (Aardal and Holm, 1995; Granger *et al.,* 2004). Saliva has the potential to cause ion suppression during LC-MS analysis in ESI through compounds in the matrix causing modifications in surface tension of the electrospray droplets and decreasing efficiency of transfer of ions in to the gas phase. Testosterone appears to be affected by ion suppression in this study and this is likely to be caused by matrix effects.

Cleaning samples with SPE can reduce ion suppression by removal of contaminating matrix compounds (Bonfiglio *et al.,* 1999). Examining SPE method development revealed the wash phase can detrimentally affect the recovery of analytes if the concentration of organic solvent elicits a non polarity above that of the analytes being extracted. Additionally, studies assessing composition of the solution to reconstitute cortisol and testosterone post SPE revealed that the percentage of organic solvent affects analyte

recovery and signal strength therefore 80% methanol is the preferred constitution. Concentration of saliva post SPE is limited due to the initial volume obtained during sampling, however concentrating samples 10 times was revealed to elicit method accuracy within the accepted criteria (<15%) for cortisol for all samples (range 2-8 ng/mL) and concentrations above 0.5 ng/mL for testosterone. Finally, recovery for both analytes was comparable with findings from similar studies and elicited consistent results therefore the SPE extraction method was accepted for use in this study.

5. LC-MS ANALYTICAL METHOD VALIDATION

Analytical method validation is designed to establish the reproducibility and accuracy of a new method and validity of its use in analyte detection and quantification. The main criteria for investigation are limits of detection and quantification, intra and inter assay precision, accuracy and linearity. The aim of this chapter is to describe the validation of the LC-MS method developed for the detection and quantification of cortisol and testosterone (Chapter 3). Part of this validation involves a comparison against a fully validated ELISA (Salimetrics, UK) for the detection and quantification of salivary cortisol and testosterone. Studies have already compared LC-MS with ELISA (Nelson *et al*., 2008) and radioimmunoassay (Jonsson *et al*., 2003) assays to measure salivary cortisol levels. Similarly, LC-MS has been compared with immunoassay techniques (Singh *et al.,* 2008; Nelson *et al.*, 2008) to measure salivary testosterone. All studies revealed correlations between the techniques.

Cortisol and testosterone levels in saliva measured during a resting state have many applications; from the diagnosis of disorders such as Cushings syndrome (Baid *et al*., 2007) and hypogonadism (Matsui *et al*., 2009) to the potential diagnosis of overtraining syndrome in athletes (Urhausen and Kindermann, 2002). Resting diurnal variation is evident for both analytes and the cortisol awakening response (CAR) can be higher in women (Clow *et al*., 2004) compared to men. Testosterone exhibits clear gender differences with men possessing higher circulating testosterone levels than women. Physical activity may also have an impact on resting hormones levels and there is evidence for an increase in resting testosterone levels in those undertaking weight training (Stone and Fry, 1997). However those undertaking prolonged high volume endurance training may exhibit a decreased resting testosterone level and higher cortisol level than untrained individuals (Gulledge and Hackney, 1996).

This chapter will evaluate the developed LC-MS method to measure cortisol and testosterone against established analytical method validation criteria. Correlations between resting salivary cortisol levels with both LC-MS and ELISA will be measured.

Furthermore, salivary cortisol and testosterone levels are examined in active versus non active men and women.

5.1 Method validation guidelines

Method validation is required for any new or amended method to ensure it can produce reproducible and reliable results and it involves satisfying a number of criteria (figure 5.01). These are based on the International Conference on harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH) guidelines (1996) and US Department of Health bioanalytical method validation guidelines (2001).

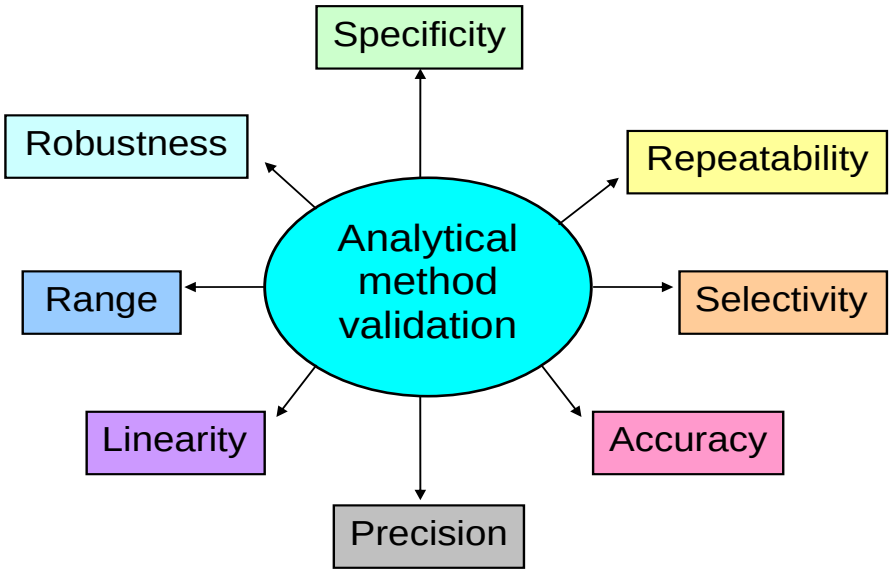


Figure 5.01 Method validation criteria (ICH, 1996)

Specificity

Specificity ensures that the signal of interest is measured despite the presence of other components such as impurities and/or degradation products. It is determined by assessing peak identity and purity.

Limit of detection and limit of quantification

The LOD is the lowest concentration of a sample that can be detected but not necessarily quantitated as an exact value; it is generally quoted as a S/N ratio of 3:1. The LOQ is the lowest amount of analyte in a sample which can be quantitated exactly and is determined by a S/N ratio of at least 5:1. It should be validated by the analysis of a number of samples know to be near the limit.

Accuracy

Accuracy is a measure of the closeness of agreement between test results and the value which is accepted as the true value. It indicates the deviation between the mean value and the true value. It should be presented as percent recovery of a known added amount of analyte in the sample. This is assessed by adding a known amount of a labelled internal standard and comparing the signal for this compound against that for the unknown concentration of analyte. Accuracy is accepted as at least 80% recovery of a known concentration at LOQ and 85% in higher samples.

Precision

Precision is a measure of the reproducibility of the whole analytical method (including sampling, sample preparation and analysis) and is presented as relative standard deviation or coefficient of variation. Precision encompasses repeatability, reproducibility and intermediate precision. Precision is accepted as a coefficient of variation <20% and <15% at the lower limit of quantification.

- Repeatability is a measure of intra assay precision (precision under the same operating conditions over a short interval of time).
- Reproducibility expresses the precision between laboratories; this is assessed by conducting an inter-laboratory trial.
- Intermediate precision expresses the precision against within-laboratory variations (e.g. different days, analysts, equipment). The method should be assessed on different days and conducted by different analysts and the variation documented.

Linearity

Linearity is the ability of a method to obtain results which are within a given range, to obtain results which are directly proportional to the amount of analyte. This is established by calculating the regression line of the results and comparing it with analyte concentration i.e. calibration standards would be run across the known range of concentrations of cortisol and testosterone and a linear regression line plotted to reveal if the samples show a proportional increase. An r^2 value of >0.95 is expected for a method to be classed as linear.

Range

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample, this is derived from linearity studies.

5.1.1 Comparison of LC-MS with ELISA

Most commonly, ELISA have been use to measure cortisol and testosterone levels in human saliva; however when measuring low levels of hormones in saliva they have a tendency to overestimate concentrations, especially for testosterone samples from women and children (Fitzgerold and Herold, 1996; Taieb *et al.,* 2003). Despite their shortcomings, ELISA show a high accuracy and precision when measuring salivary cortisol and previous researchers have shown a correlation with LC-MS of 0.63 (Nelson *et al*., 2003). Nelson and co-workers also reported higher accuracy in LC-MS compared to ELISA. Higher correlations have been found when comparing testosterone samples measured with LC-MS and ELISA (r=0.81, Yasuda *et al.,* 2008). As previously mentioned, correlational analysis has been commonly used to compare the relationship between methods, however limits of agreement may give a more accurate indication of how closely two sets of values agree (Bland and Altman, 1986).

5.1.2 Salivary measures at rest

Advantages of measuring salivary hormones include monitoring diurnal changes and diagnosing some diseases. A further application is the analysis of resting sample from athletes as changes in the ratio of cortisol to testosterone has been hypothesised as an indicator of overtraining syndrome (Urhausen and Kindermann, 2002).

Gender differences have been identified in resting hormones such as lower testosterone in men and there are also reports that the cortisol awakening response (CAR) can be affected by gender with evidence that women elicit a higher response than men (Pruessner *et al.,* 1997; Clow *et al.,* 2004; Weekes *et al.,* 2008). Resting hormone concentrations may also be affected by exercise status. There is evidence that individuals undertaking prolonged hard endurance training show a reduction in resting testosterone levels and increase in circulating cortisol levels (Vervoorn *et al.,* 1991**;** Elloumi *et al.,* 2003). This catabolic process may indicate the breakdown and repair of muscle tissue.

This chapter aims to assess accuracy and precision of ELISA compared to LC-MS for measurement of salivary cortisol levels and evaluate the correlation between the two measurements with saliva samples collected from active and non active men and women. Concentrations of salivary cortisol and testosterone from these groups will also be examined to establish whether resting salivary measurements at rest differ between sedentary and active participants.

5.2 Methods

5.2.1 Sample extraction and LC-MS

Cortisol and testosterone were extracted with SPE and samples analysed with LC-MS employing the method described in chapter 2.

5.2.2 Method validation

For method validation the following approach was followed:

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were established by analysing six calibration samples in triplicate. Cortisol standards were prepared in HPLC grade water (0, 1, 2, 5, 8 and 10 ng/mL, 3 ng/mL IS), underwent extraction SPE (as described in Chapter 4) and were concentrated 10 times prior to analysis. Testosterone calibration standards (0, 0.1, 0.2, 0,5, 0,8 and 1 ng/mL, 0.3 ng/mL IS and 0.0, 0.1, 0.2, 0.5 and 1.0 ng/mL, 0.3 ng/mL IS) also underwent SPE and concentration prior to analysis. Samples were analysed with LC-MS and their S/N ratio plotted against concentration. The regression equation produced from this calibration curve was used to extrapolate the S/N corresponding to LOD and LOQ. S/N ratio of the cortisol and testosterone peaks corresponded to either LOD (S/N ratio = 3) or LOQ (S/N ratio = 5). The experiment was repeated on three separate days.

Repeatability

High, medium and low concentration solutions were prepared for cortisol (2 ng/mL, 5 ng/mL and 8 ng/mL) and testosterone (0.2, 0.5 and 0.8 ng/mL). Aliquots of these solutions were repeatedly (n=5) measured on the same day, and the coefficient of variation (%CV) calculated to establish how reproducible the measurements were across the reference range of each analyte. Mean peak area of each concentration was calculated and divided by the

standard deviation of the samples with the following equation [mean peak area /standard deviation*100].

Inter assay precision

High, medium and low concentration solutions were prepared for cortisol and testosterone (as above). Aliquots of these solutions were repeatedly (n=5) measured on two separate days and the coefficient of variation (%CV) calculated to establish how reproducible the measurements were across the reference range of each analyte. Inter assay precision (% CV) was determined by calculating the mean peak area for each concentration on both days and these means were combined to give an average across the days then the standard deviation between then was calculated. Inter assay precision for each concentration was calculated by dividing the standard deviation between day 1 and 2 by the mean peak area for both days and multiplying by 100 to give a percentage [mean peak area day one and two/standard deviation between days*100].

Linearity

A range of cortisol and testosterone calibration standards (0, 1, 2, 5, and 10 ng/mL, 3ng/mL IS) and further testosterone standards (0, 0.1, 0.2, 0.5 and 1 ng/mL; 0.3 ng/mL IS) were tested for linearity on three occasions (n=3). Peak area ratio between the analyte and internal standard was plotted graphically against concentration and the regression line established. Linearity was accepted with an r^2 value > 0.95.

Accuracy

Accuracy was determined for each three known concentration of cortisol (2. 7 and 8 ng/mL) and testosterone (0.2, 0.3 and 0.8 ng/mL) Cortisol and testosterone were extracted with SPE, dried and reconstituted before analysis with LC-MS. The measured amount is divided by the expected amount and multiplied by 100 to express as a percentage (equation 4) [% accuracy = (measured/known)*100].

5.2.3 Method comparisons

Recruitment

For study one, twenty men and five women aged 18-55 were recruited from running clubs and the local university community. Before the study all participants were given an

information sheet and the opportunity to answer any questions about the study. They also completed a health questionnaire (Appendix B), and signed an informed consent form (Appendix A). For study two, the same protocol for recruitment was used however 19 men and 11 women aged 18-55 were recruited, some participants took part in both studies. Exclusion criteria included taking any prolonged medication or oral contraceptives.

Participants

Male participants were divided into active and sedentary groups according to their level of exercise. "Active" was classified as participating in organised physical activity for a total of >3 hours of physical activity per week. Women in the study were a mixture of active and non active. Physical characteristics for participants can be seen in table 5.1 and study two in table 5.2.

Table 5.1 Participant characteristics (mean ± SD) (study one)

	Non active men	Active men	Women
Age (y)	35 ± 7	33 ± 8	35 ± 11
Body Mass (kg)	77.2 ± 13.9	70.0 ± 4.9	65.6 ±9.7
Physical activity (h.wk)	1.3 ± 0.8	6.2 ± 2.0	2.4 ± 1.8

Table 5.2 Participant characteristics (mean ± SD) (study two)

	Non active men	Active men	Women
Age (y)	36 ± 9	34 ± 9	34 ± 9
Body Mass (kg)	79.9 ± 13.1	73.4 ±4.9	62.3 ±9.4
Physical activity (h.wk)	0.8 ± 0.5	5.8 ± 2.5	3.7 ± 2.0

Sample collection and storage

Saliva collection and storage was performed as described in Chapter 2. Samples took between 5 and 15 minutes to produce.

Sample analysis

ELISA

Samples were thawed and analysed for cortisol and testosterone as described in chapter 2. The intra assay CV for ELISA was 10.1 ± 5.8%.

Extraction and LC-MS

Cortisol and testosterone were extracted with SPE and analysed for cortisol with LC-MS employing the method described in chapter 2. A calibration curve was prepared for each study (table 2.1, Chapter 2) and samples quantified according to peak area ratio using the regression equation established from the calibration. The intra assay CV was 11.0 ± 8.5% for MS.

5.2.4 LC-MS (with and without SPE) compared to ELISA

Calibration curves and known amounts of cortisol (0, 1, 2, 5, 8 and 10 ng/mL, 3 ng/mL IS) were prepared with or without SPE and compared for accuracy and recovery with LC-MS (with and without extraction) and ELISA. Samples (1000 µL) were either dried with a vacuum centrifuge and reconstituted in 100 µL mobile phase (MeOH: water, 50:50) and analysed with LC-MS (described in chapter 2) or samples (1000 µL) were diluted with water (1000 µL) extracted with SPE (Chapter 2), dried and reconstituted (100 µL) for analysis. Samples (1000 µL) were also prepared without IS and analysed for cortisol with ELISA as described in chapter 2. The mean intra assay coefficient of variation was 7.52%. Recovery of the LC-MS samples was calculated by the following equation [Mean peak area of SPE sample/peak area non SPE sample*100]. For ELISA the amount of the control samples recovered compared to the expected amount was calculated as a percentage.

5.2.5 Statistical analysis

Statistical analysis was performed using SPSS (Version 18.0, Champaign, ILL, USA). Data was checked for normality prior to statistical analysis. Pearson's correlation coefficient was used to compare cortisol and testosterone for ELISA and LC-MS. Limits of agreement were also used to evaluate the agreement between the two methods. A one way ANOVA with post hoc Bonferoni tests and independent t-tests were used to compared the cortisol and testosterone levels between the different groups. A P value of <0.05 was accepted as significant.

5.3 Results and discussion

5.31 LOD and LOQ

S/N ratio of the concentrations of cortisol and testosterone in a calibration curve (figure 5.02) were used to establish LOD and LOQ for each analyte and are presented in table 5.3.

The LOQ of cortisol was 0.6 ng/mL. Quantification of cortisol was achieved within the accepted range for measurement in saliva (1-8 ng/mL; Aardal and Holm, 1995) however for testosterone the LOQ was 273 pg/mL; this is above the resting reference range for men (49-190 pg/mL) and the LOD was 102 pg/mL. Therefore, in resting saliva samples some testosterone peaks could be detected however the S/N ratio was not sufficient to quantify this analyte and the LOQ for testosterone was outside the lower end of the reference range for men by a factor of 5.6. Samples were concentrated 10 times and further concentration revealed no improvements in accuracy (chapter 4) and was not viable due to sample volume restrictions during exercise which was the target application. Despite the inability to accurately quantify testosterone, peaks were visible in a male saliva sample; however the S/N ratio was below that required for quantification due to background noise (figure 5.03).

Table 5.3 LOD and LOQ for cortisol and testosterone using LC-MS (n=4)

Mean ± SD	Cortisol (ng/mL)	Testosterone (pg/mL)
LOD	0.31 ± 0.007	102 ± 8
LOQ	0.60 ± 0.042	273 ± 17

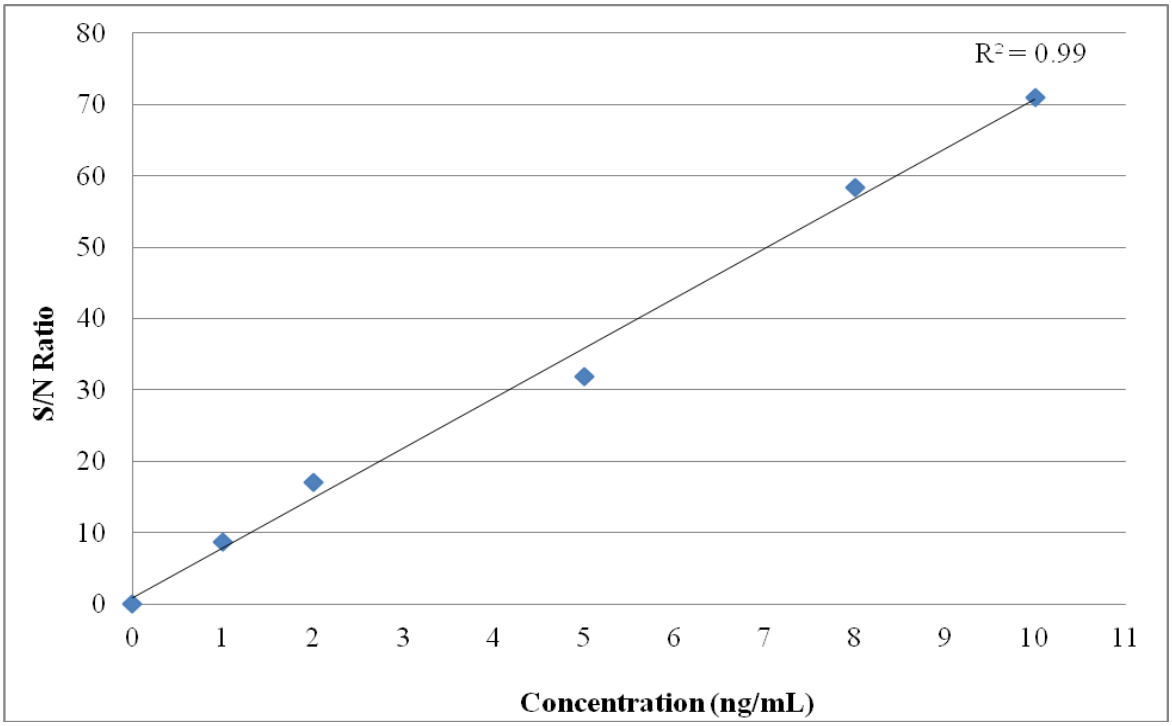


Figure 5.02 Example of the S/N ratio calibration to establish LOD (S/N=3) and LOQ (S/N=5) of cortisol.

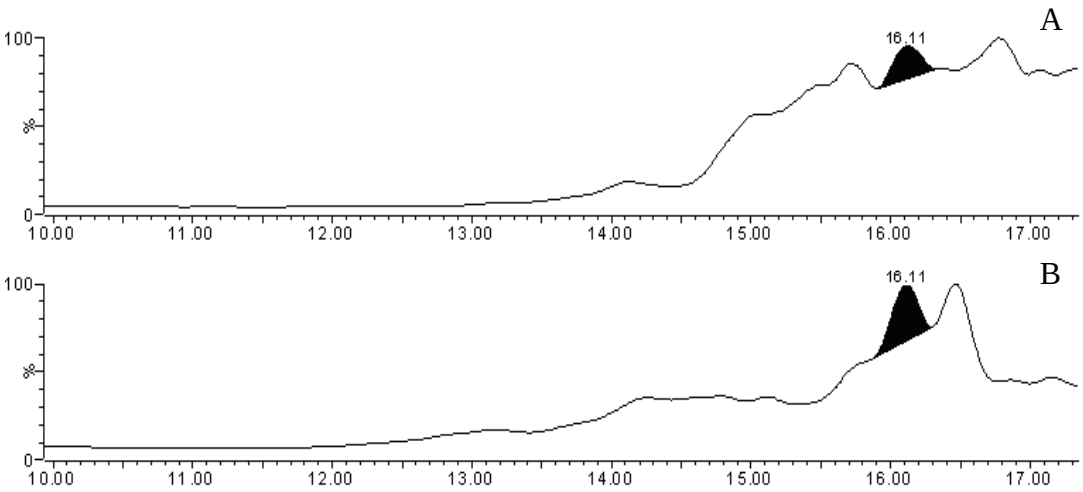


Figure 5.03 Male saliva sample displaying shaded peaks for testosterone ([A], *m/z* 289.4 RT 16.11 mins) and d_3 testosterone ([B], *m/z* 292.4 RT 16.11 mins)

In this study, LOQ is below levels achieved by most other research groups measuring salivary cortisol levels; one study of LC-MS revealing a LOQ of 0.017 ng/mL (Katoaka *et al.*,2007) for cortisol and LOQ has ranged from 0.017 ng/mL to 0.72 ng/mL (Owen *et al.*,2010). The present study reported a LOQ close to the upper limit LOQ in the literature. There are few research methods available for detection and quantification of testosterone in saliva, probably due to the trace levels in this medium. Recent methods utilising LC-MS/MS have reported LOQ of 0.005 ng/mL (Kutsukake *et al.*, 2009; Matsui *et al.*, 2009). Both studies employed an API 4000 electro spray ionization (ESI) mass spectrometer (Applied Biosystems, USA) The specification of the MS machine used in this work is likely to contribute to the quantification limit falling at high end of the range achieved in other studies as detectors and ESI interfaces have improved greatly for detection and quantification of small molecules in the past 10 years. The API 4000 has been found to elicit an 8 fold improvement in sensitivity compared to the API 3000 (Kushnir *et al.*, 2006). This is because the API 4000 possesses improved gas dynamics (i.e. transfer from liquid to gas phase ions) leading to increased ion yield in the gas phase, which has the potential to elicit a 10 fold increase in sensitivity (ABSciex, 2010). This exemplifies how mass spectrometer specifications can impact on quantification limits in an LC-MS method.

Sample quantification

Calibration samples were prepared and quantified by adding an internal standard prior to SPE. The ratio between the peak area of analyte and the internal standard were used to prepare a calibration using the labelled standard method of quantification described in chapter 1. This method was chosen above standard addition due to the lower volume of sample required and shorter preparation and analysis time. Examples of calibration curves for cortisol and testosterone with resultant regression equations are illustrated in figures 5.04 and 5.05 respectively.

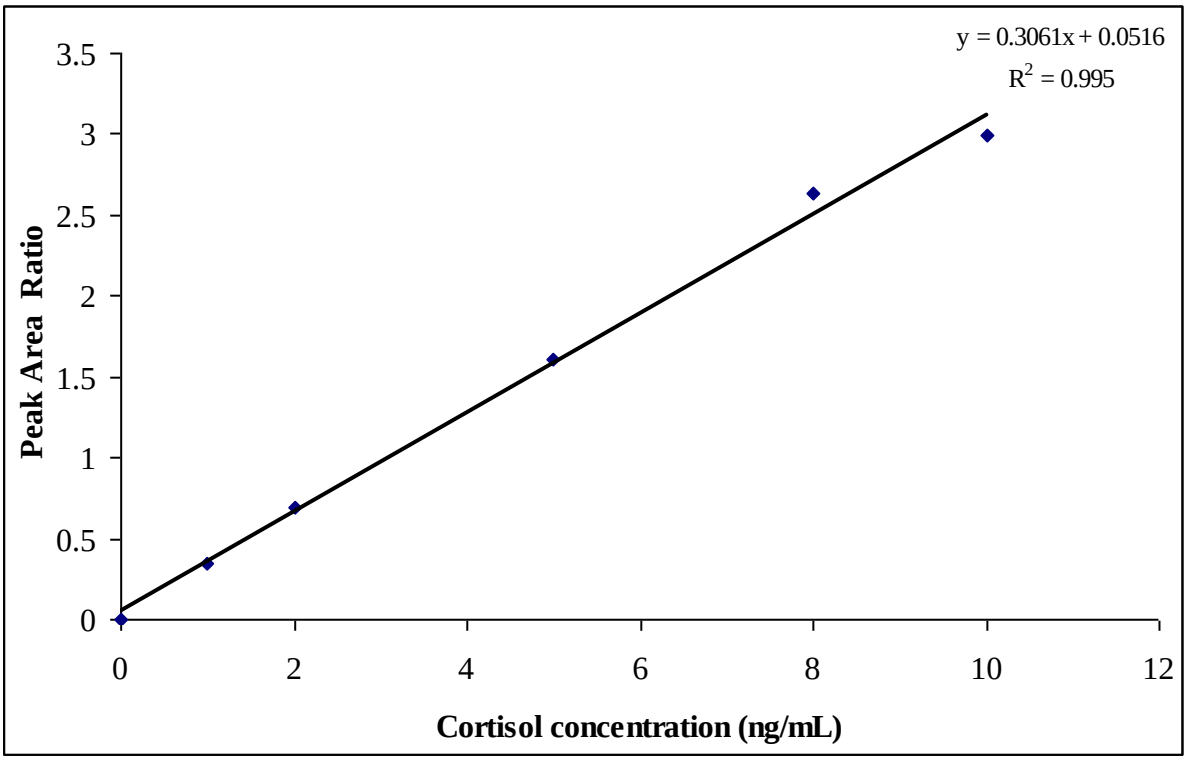


Figure 5.04 Calibration graph (0-10 ng/mL cortisol) illustrating peak area ratio of cortisol and deterated (d_2) cortisol against concentration

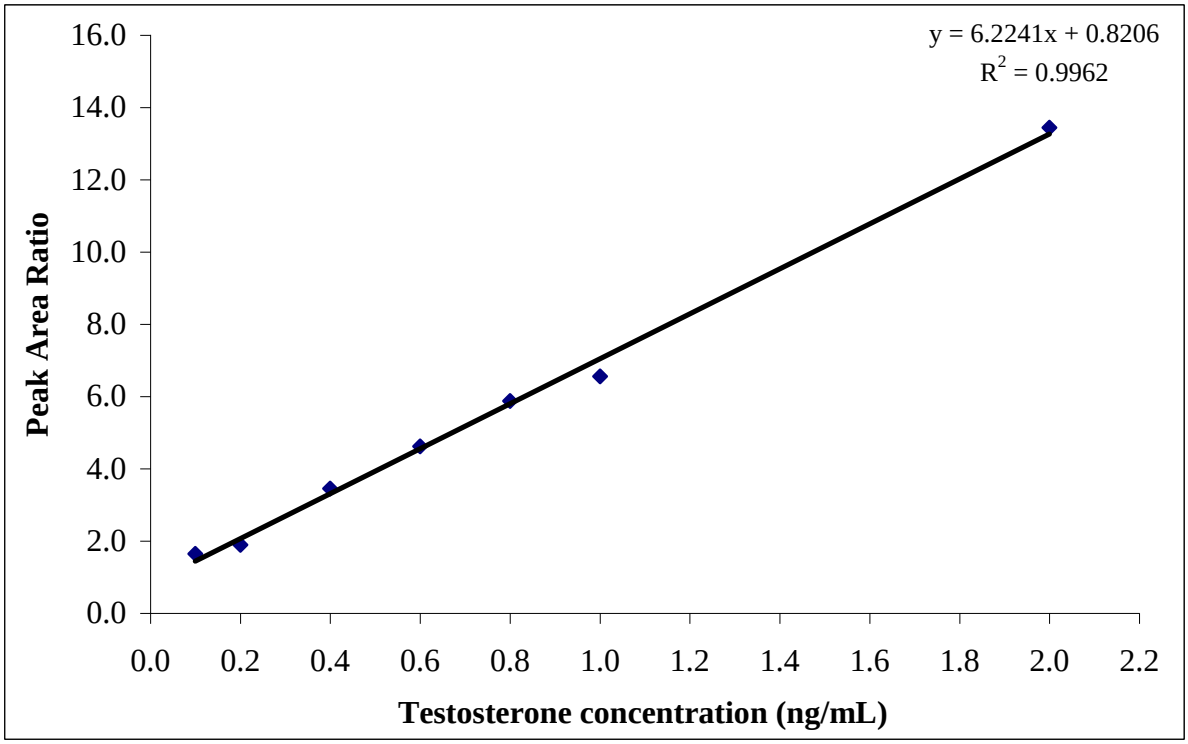


Figure 5.05 Calibration graph (0.1-2 ng/mL testosterone) illustrating peak area ratio of testosteorne and deterated (d_3) testosterone against concentration

Testosterone calibration samples were prepared in the range 0.1-2 ng/mL as this was representative of the reference range for salivary testosterone in men, whereas for cortisol the concentration in saliva is 10 fold higher. Addition of an internal standard controlled for fluctuations in ionisation and analyte detection between runs linked to the mass spectrometer. Adding a labelled standard prior to cleaning also control for any analyte loss during sample cleaning (Cawood *et al.,* 2005; Jonsson *et al*., 2003) and can improve analytical method accuracy.

5.32 Precision

Repeatability (intra day)

Repeatability or intra assay precision of three concentrations of cortisol and testosterone repeated 10 times was found to be higher at the lower reference range of quantification than the upper range (table 5.5). The mean CV was 6.72% for cortisol and 8.15% for testosterone. The accepted validation criteria for repeatability is a CV of <20% (US Department of Health, 2001), therefore both cortisol and testosterone met the desired criteria for precision. The present study reports intra assay precision of <10% for both cortisol and testosterone. Other studies have reported intra assay values for salivary

cortisol of 15.8% (Kutsukake *et al.*,2009); 7.0% (Jonsson *et al.*,2003) and 5.1% (Li *et al.*, 2008) therefore the current method is comparable. Similarly, testosterone precision in the current method validation is comparable to those reported in other studies with intra assay values of 10.9% (Kutsukake *et al.*, 2009) and 7.0% (Matsui *et al.*, 2009). Intra assay precision is affected by variation in injection, sample preparation, instrument parameters and matrix effects and the primary reason for adding an internal standard is to control for these fluctuations (Magni *et al.*, 2001).

Table 5.4 Repeatability/intra day and inter day precision for cortisol (2. 5 and 8 ng/mL) and testosterone (0.2, 0.5 and 0.8 ng/mL) (n=5) (%CV)

Cortisol concentration (ng/mL)	CV (%)		Testosterone concentration (ng/mL)	CV (%)	
	Intra assy	Inter assay		Intra assay	Inter assay
2	8.67	6.93	0.2	9.38	6.61
5	7.22	3.90	0.5	8.06	2.01
8	4.28	6.60	0.8	7.02	6.52

Inter day precision

Inter day precision was established from repetition of three concentrations of cortisol and testosterone on two separate days (table 5.4). Mean inter day precision was 5.80% for cortisol and 5.04% for testosterone. The present study reports inter assay precision of 5.80% for cortisol which is comparable with other studies quantifying salivary cortisol levels. Findings of 5.8% (Kutsukake *et al.*, 2009); 11.0% (Jonsson *et al.*, 2003) and 1.7% (Li *et al.*, 2008) have been reported in the literature. Similarly for testosterone intra assay precision in this study was 5.04% and compares with 6.2% (Kutsukake *et al.*, 2009) and 1.0% (Matsui *et al.*, 2009). Inter assay or between day precision can be affected by inter day variations in the mass spectrometer and conditions such as temperature and sample handling procedures.

5.3.3 Accuracy

Accuracy is the comparison of the measured compared to the expected amount of analyte. Results are presented in Chapter 4 (table 4.2, 10% concentration). The mean accuracy across the range of samples tested was 6.04% for cortisol and 12.69% for testosterone. These results are accepted in method validation and are similar to other LC-MS studies

which have reported accuracies of 9.5% (Kutsukake *et al.,* 2009), 2.2% (Matsui *et al.,* 2009) and 9.0% (Jonsson *et al.,* 2003) for cortisol. However, for testosterone accuracy is above values levels achieved by other studies such as 2.4% (Kutsukake *et al.,* 2009) and 2.5% (Matsui *et al.,* 2009). Compounds co-eluting with the chosen analyte can affect accuracy of an LC-MS assay. Also inconsistency during sample preparation may affect method accuracy, although addition of an internal standard in this method serves to negate these errors.

5.3.4 Linearity

Cortisol calibration samples tested on three separate occasions revealed an average r^2 value of 0.991 and a mean r^2 value of 0.975 was achieved for testosterone. Linearity is accepted with an r^2 value of > 0.95 (ICH, 1996) indicating this method can be accepted as linear in the tested range (0-10 ng/mL for cortisol and testosterone). Testosterone was also examined with a lower calibration range (between 0 and 1 ng/mL) and this exhibited an r^2 value of 0.998 which suggests the method is also linear in this lower range for testosterone. The addition of an internal standard to control for ionisation suppression can potentially improve linearity (Liang *et al.,* 2003). Liang and colleagues found adding an internal standard of a concentration appropriate to the assay lead to mutual suppression of the IS and analyte signal. Howver, they also observed that if IS concentration is too high this can suppress the analyte signal during ESI and affect linearity, accuracy and reproducibility of the calibration curve,

5.3.5 Comparison study

In this study only cortisol is quantified due to the LOQ of testosterone falling below the established reference range in saliva. Stock samples measured with ELISA and MS were compared with and without SPE. Findings revealed there was high linearity for a set of cortisol calibration samples using both ELISA and LC-MS with and without SPE (0-10 ng/mL; figure 5.06). Accuracy, intra assay precision (CV) and sample recovery are presented in table 5.5.

Table 5.5 Cortisol accuracy (%), CV (%) and recovery (%) (n=3) determined by ELISA LC-MS (SPE and no SPE)

Technique	Accuracy (%)	Precision (%CV)	Recovery
ELISA	94.6 ± 4.2	7.2 ± 11.3	100.4 ± 7.2
MS (no SPE)	91.5 ± 6.0	2.0 ± 1.9	N/A
MS (SPE)	88.4 ± 8.0	6.5 ± 9.1	85.4 ± 0.04

ELISA showed higher accuracy than LC-MS however precision (%CV) was lower with LC-MS. ELISA sample recovery was higher than LC-MS with SPE however cortisol recovery achieved with LC-MS is comparable with acceptable levels found in other MS studies previously described (Chapter 4).

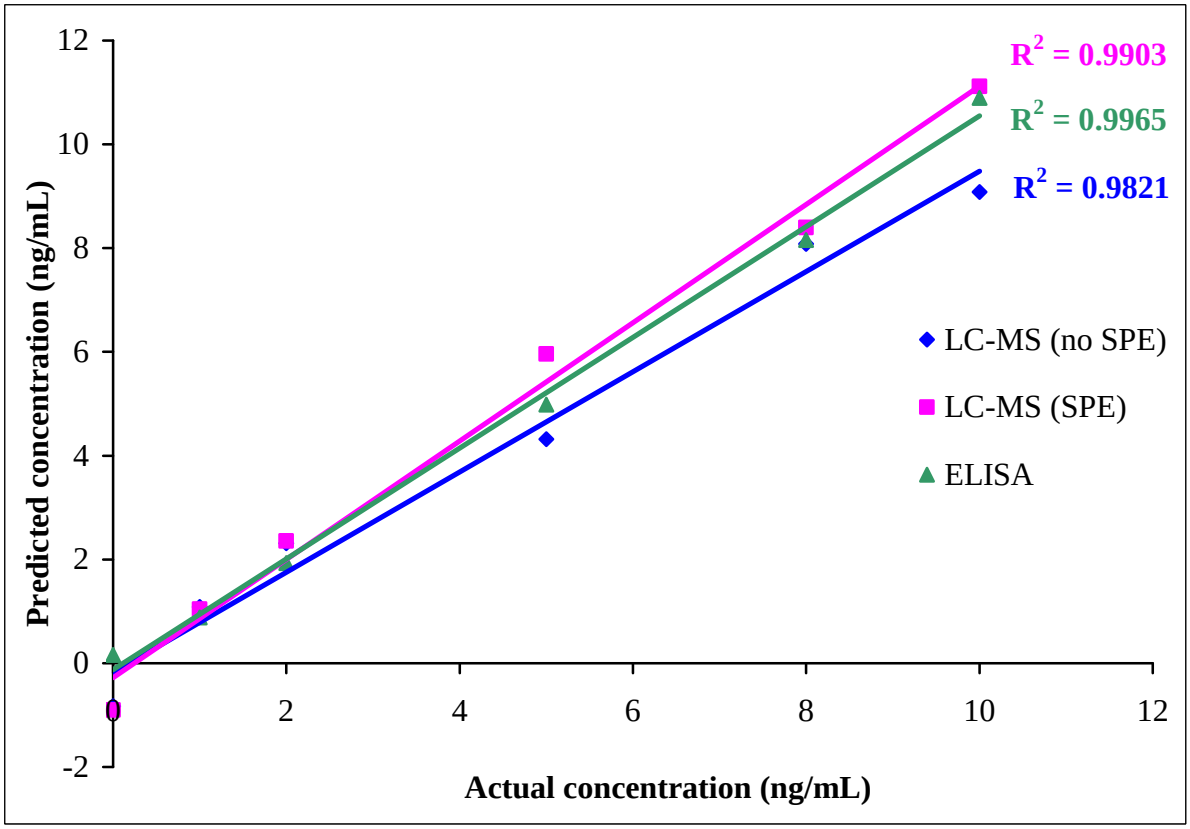


Figure 5.06 Validation of cortisol comparing ELISA and MS (with and without SPE) (n=6)

5.3.6 Method validation – samples collected at rest

Resting study one

The aim of the study was two fold 1) to validate the LC-MS method for cortisol and testosterone and 2) investigate if there was a difference between active and non active men and women (mixed activity status).

Participant characteristics are presented in table 6.4. ELISA results for cortisol and testosterone are presented in table 5.1.

Table 5.6 Mean ± SD salivary cortisol and testosterone for active men (n=10) and non-active men (n=10), all men (n=20) and women (n=5) determined by ELISA

Mean ± SD	Active	Non active	All men	Women
Cortisol (ng/mL)	3.7 ± 1.6	3.8 ± 1.9	3.7 ± 1.7	4.8 ± 2.7
Testosterone (pg/mL)	168.0 ± 57.7	231.9 ± 126.2	199.9 ± 105.5	101.5 ± 77.5

There was a correlation between saliva samples (n=22) measured with ELISA and LC-MS ($r=0.83$, $p<0.001$, figure 5.07). Three samples were omitted due to high viscosity causing issues during SPE. When comparing MS and ELISA. The mean difference between the methods was -0.54 ng/mL and limit of agreement between MS and ELISA was 4.23 ng/mL below and 3.16 ng/mL above. This showed poor agreement of ELISA with MS, however the results were correlated.

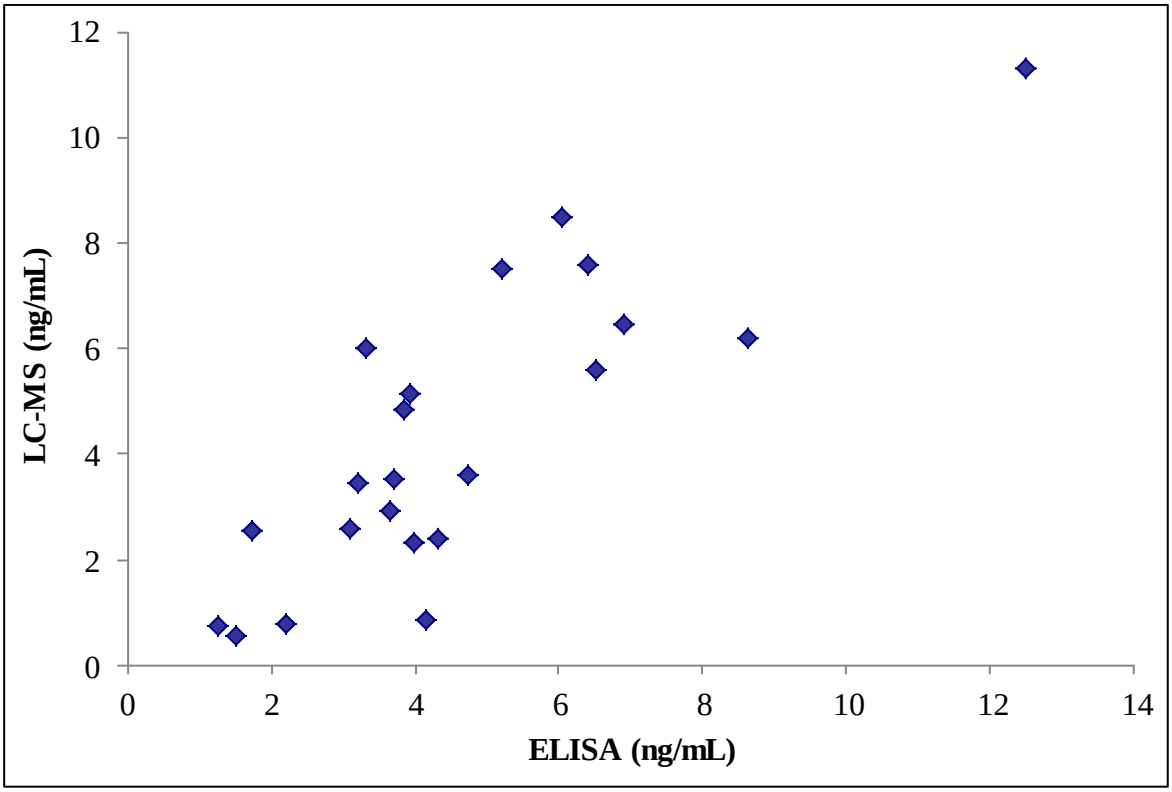


Figure 5.07 Comparison of cortisol samples (n=22) with ELISA and LC-MS

Comparison of hormone levels measured with ELISA revealed there was no significant difference between active and non-active men for levels of cortisol or testosterone (table 5.6). However an independent t-test showed a trend towards lower testosterone in women compared to men (p=0.055).

Resting study two

Resting saliva samples were collected to repeat the protocol used in study one as it was identified that the speed of elution during the SPE procedure may have contributed to a loss of analyte. This second study also included a higher number of women, improving statistical power when comparing their hormone levels with men. Participant characteristics are presented in table 5.2.

Correlation analysis comparing ELISA and LC-MS revealed a strong correlation (r=0.80, p=0.0001, figure 5.08). This was marginally higher than study one. Results from both studies reveal that the LC-MS method is accurate, precise and linear and is comparable with ELISA analysis therefore this method is validated for use in salivary analysis of cortisol. The mean difference between the methods was 1.64 ng/mL and limit of agreement between MS and ELISA was 0.79 ng/mL below and 4.08 ng/mL above. This showed poor agreement of ELISA with MS likely to overestimate results; however the results are correlated and show a similar trend.

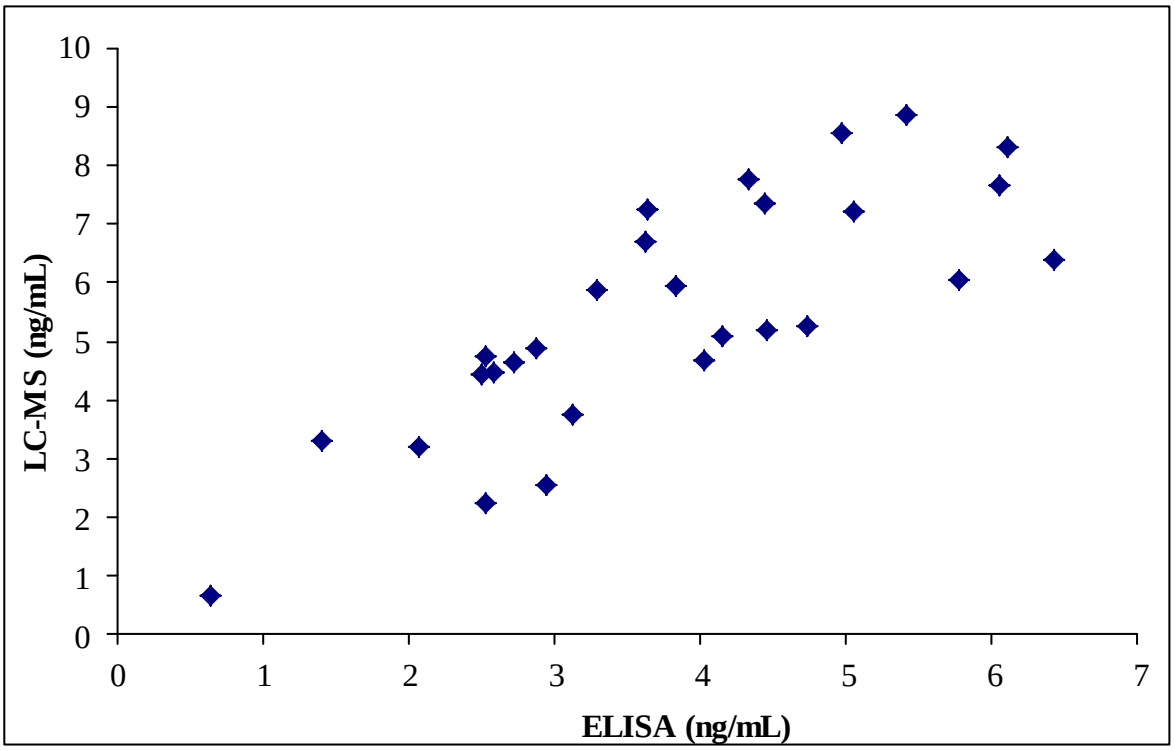


Figure 5.08 ELISA vs. LC-MS for resting cortisol samples (n=29)

Group comparisons revealed no significant difference between active and non-active men for cortisol or testosterone concentrations however gender differences were observed with cortisol levels significantly higher in women (p=0.004) and testosterone levels significantly lower compared to all men (p=0.0001; table 5.7). There is evidence to suggest that the cortisol awakening response (CAR) is higher in women (Pruessner *et al.*, 1997; Clow *et al.*, 2004; Weekes *et al.*, 2008). Additionally, psychosomatic stress has been shown to potentially increase the CAR (Wust *et al.*, 2000; Schultz *et al.*, 1998).

Table 5.7 Mean ± SD salivary cortisol and testosterone levels for active men (n=9) and non-active men (n=9), all men (n=18) and women (n=11) measured with ELISA.

Mean ± SD	Active	Non active	All men	Women
Cortisol (ng/mL)	3.6 ± 1.5	3.0 ± 0.9	3.3 ± 1.3	4.9 ± 1.5
Testosterone (pg/mL)	160.7 ± 38.0	148.8 ± 53.4	154.7 ± 45.4	81.8 ± 32.9

5.4 Summary

Method validation procedures suggest the present method credible for salivary cortisol quantification within the reference range (0-10 ng/mL; Aardal and Holm, 1995). The

method meets the ICH (1996) and US Department of Health (2001) criteria for accuracy, intra and inter assay precision and linearity. However the testosterone LC-MS assay falls outside the reference range required to quantify this analyte in male and female saliva, despite the ability to detect testosterone in some samples. The mass spectrometer may be a limiting factor in the quantification of testosterone as newer ESI interfaces eliciting more efficient ionisation have the potential to increase assay sensitivity up to 10 fold (Kushnir *et al*., 2006). Concentration of samples in Chapter 4 revealed no increase in accuracy in the reference range for testosterone and further concentration is not possible due to restrictions on sample volume during exercise. To monitor the acute response at multiple time points it is feasible to collect a sample of 2-3 mL within a 3 minute collection period based on salivary flow rate (Heintze *et al.,* 1983). A further aim was to compare LC-MS with ELISA to quantify salivary cortisol levels in resting samples and results revealed a strong correlation between methods which is comparable with other studies comparing measurement of cortisol with these techniques (Jonsson *et al*., 2003). Limits of agreement analysis showed poor agreement between absolute values but given the difference in techniques this is expected.

Finally, a comparison of resting salivary cortisol and testosterone levels measured with ELISA revealed that women showed higher salivary cortisol levels than men and this may be due to an enhanced CAR observed in this population (Clow *et al*., 2004). Resting cortisol and testosterone concentration did not differ between active and sedentary men and the results may be explained by the level of exercise in the active group being insufficient to elicit a prolonged or chronic alteration in these hormones.

6. CORTISOL AND TESTOSTERONE RESPONSE TO ACUTE EXERCISE

6.1 Introduction

Physical stress such as exercise stress mobiles the endocrine system. The hypothalamus is activated after the onset of a stressor and secretes ARH which stimulates the pituitary gland to secrete ACTH. This hormone in turn stimulates the adrenal glands to release stress hormones such as adrenaline and cortisol. Activation of the sympathetic nervous system leads to the short term release of catecholamines into the circulation (adrenaline and noadrenaline) followed by the release of cortisol which is slower and can be more prolonged. Acute stress has also been shown to increase circulating levels of testosterone (Sutton *et al.,* 1973) and activation of catecholamines may be one of the main mechanisms stimulating secretion of testosterone during stress (Chrousos, 1998). However the exact mechanisms for the increase in testosterone are contentious. The extent of the endocrine response is dependent on the intensity and duration of the exercise stimulus. Cortisol and testosterone are markers of the exercise stress response and also acknowledged for their potential as useful biomarkers in the diagnosis of overtraining syndrome (Urhausen and Kindermann, 2002). Currently, quantification of these hormones in exercise studies is predominantly through ELISA however MS is emerging as an alternative way of measuring these hormones.

To date, many studies have investigated the effect of exercise on cortisol following acute exercise with most reporting increases following acute continuous exercise (Jacks *et al.,* 2002; Allgrove *et al.,* 2008; Budde *et al.,* 2010; O'Connor and Corrigan, 1987; Rudolph and McCauley, 1998). The overall consensus supports the proposal of a 'threshold' of exercise intensity above which cortisol levels increase and this is suggested to be at least 20 mins of exercise above 60% VO_{2max}. It has also been suggested that when exercise is performed under 60% VO_{2max}, hormonal responses will only occur when a certain amount of work is done i.e. running for an adequate time (Viru *et al.,* 1992). Tremblay *et al.,* (2005) demonstrated this to be 80 minutes of running at low intensity (55% VO_{2max}). Furthermore, some researchers have examined intermittent exercise and support the hypothesis of the intensity threshold (Hough *et al.,* 2011).

The response of testosterone to acute exercise also appears to be affected by a threshold intensity required to elicit an increase (Budde *et al.,* 2010; Kokalas *et al*., 2004); however the bounds of this threshold are yet to be established. Conversely it has been identified that prolonged intense exercise such as marathon running elicits a decrease in testosterone levels (Marinelli *et al.,* 1994; Franca *et al.,* 2006; Karkoulias *et al.,* 2008; Brownlee *et al.,* 2005). The high physiological stress of a marathon may contribute to this decrease, and research has shown testosterone levels may be related to cortisol release; therefore a significantly high increase in cortisol may lead to a subsequent decline in testosterone levels (Brownlee *et al.,* 2005).

It is also possible that athletes may become accustomed to performing a certain type and intensity of exercise and therefore adapt (Vuorimaa *et al.,* 2008) requiring extra stress to elicit a hormone response; supported by studies by Moreira *et al.,* (2009) and Eliakim *et al.,* (2009). The latter two studies were conducted during a training session therefore exercise intensity was not controlled. These studies offer an insight into cortisol responses in real situations. Furthermore, the majority of studies have been conducted outside a laboratory setting, therefore overall intensity is unknown. Despite many studies examining the response of cortisol and testosterone to exercise, there are few comparing both cortisol and testosterone responses to different types of exercise training e.g. intermittent and continuous sessions in one group of individuals (Hough *et al.,* 2011; Vuorimaa *et al.,* 2008). Further studies could add insight into training responses and hormonal response. Additionally, there have been no studies examining the cortisol and testosterone response to aerobic circuit training; a common training mode utilised by many athletes.

Despite no studies on aerobic circuit training there has been research investigating the effect of structured weight training on cortisol and testosterone. Those who observed an increase in testosterone suggested the magnitude of stress of the session or intensity may play a part. In their recent review, Vingren *et al.,* (2010) proposed there is a threshold of intensity to elicit a testosterone response to resistance training (supported by studies from Yarrow *et al.,* 2007; Kraemer *et al.,* 1990; Raastad *et al.,* 2000; Hakkinen *et al.,* 1993, Linnamo *et al.,* 2005). Training volume (total workload performed) also appears to be important with a higher volume eliciting a greater metabolic demand. Moreover, Similios *et al.,* (2003) reported that a higher volume of training led to a greater testosterone

response. However some studies failed to observe an increase after resistance training (Beaven *et al.,* 2008; Kokalas *et al.,* 2004). The literature presents no clear trend regarding cortisol response to resistance exercise. The overall consensus is that the adaptive response to strength training is likely to be multi faceted with several acute training factors (one of them hormonal) rather than a single factor.

Plasma cortisol and testosterone measures can be taken from blood and saliva, studies have shown intravenous injection of these hormones elicits immediate detection in saliva for cortisol (Kirschbaum and Hellhammer, 2000; Wang *et al., 1*981) and testosterone (Vittek *et al., 1*985; Wang *et al., 1*981). Though studies examining the hormonal exercise response have reported delays of up to 30 minutes for peak cortisol levels in saliva after exercise (O'Connor and Corrigan, 1987; Crewther *et al.,* 2008; Hough *et al., 2*011; Daly *et al., 2*005). Delays are also evident for peak salivary testosterone however these are shorter than cortisol; occurring within 10 mins of exercise cessation (Hough *et al., 2*011; Daly *et al., 2*005). These results suggest that exercise may affect diffusion of cortisol from blood into saliva; this is suggested to be due to increased sympathetic activity during intense exercise which causes vasoconstriction in the arterioles that supply the salivary glands, resulting in lower salivary volume (Chicharro *et al*., 1998). The reduced blood flow may explain the delay in cortisol and testosterone delivery and diffusion from blood into the saliva found after acute exercise.

In previous studies, salivary and plasma cortisol and testosterone concentrations were measured with ELISA or radioimmunoassay (Chapter 1, table 1.5 and 1.6) however MS is now commonly used to quantify these hormones in blood (Fanelli *et al.,* 2010; Cawood *et al.,* 2005; Jonsson *et al.,* 2003) and saliva (Kutsukake *et al.,* 2009; Matsui *et al.,* 2009). Also when comparing MS to ELISA assays the latter have been shown to lack sufficient sensitivity and specificity to measure low levels of steroids such as testosterone in women (Taieb *et al.,* 2003; Herold and Fitzgerold, 2003). MS is already used routinely in hospitals and to test individuals' testosterone levels during doping control (Shackleton *et al.,* 1997, Thevis *et al.,* 2005). Given the increased automation of MS this technique would be beneficial for use in exercise research to measure multiple hormones quickly and accurately.

The aim of this chapter are to examine the salivary cortisol and testosterone response to three different exercise bouts in runners, and will investigate correlations between salivary and blood measures taken simultaneously. Furthermore, the LC-MS method developed in earlier chapters of this thesis will be used to measure salivary cortisol and testosterone levels and validate against ELISA measurements.

6.2 Methods

6.2.1. Participants

Participants were recruited by emailing local athletics and running clubs. 13 healthy male runners agreed to participate in the study and 10 completed the study. Participants competed regularly in running, triathlon and ironman competitions and trained 4-8 times per week. Participant characteristics are presented in table 6.1. Ethical approval was granted from the university ethics committee. Prior to commencing the study participants received written and verbal instructions via information sheet (appendix D) detailing what the study entailed, and they also completed a health questionnaire (appendix B) and gave their written informed consent (appendix A).

Table 6.1 Participant characteristics

Variable	Mean (± SD)
Age (y)	39.3 ± 6.6
Body mass (kg)	76.6 ± 8.7
Height (m)	1.78 ± 0.06
VO_{2max} (ml.kg.min)	59.2 ± 5.9
Maximum heart rate (bpm)	180 ± 11

6.2.2 Experimental procedures

All participants reported to the laboratory on five occasions. Participants visited the laboratory between 3pm and 8pm for this trial as cortisol and testosterone levels show better stability at this time (Rose, *et al., 1*972). Participants were asked to refrain from eating and consuming caffeine three hours before each trial and from strenuous exercise and alcohol consumption in the 24 hours before each trial.

Maximal oxygen uptake

On the first visit to the laboratory participants undertook a VO_{2max} test on a pre-programmed treadmill (Woodway ELG55). In order to establish participants lactate threshold during the VO_{2max} test an incremental speed protocol with interruptions was employed (similar to Vuorimaa *et al., 2*008). Prior to starting the test resting lactate measures were taken via a finger tip blood sample (20 µL) and the sample was mixed in a pre filled reaction cup (EFK Diagnostic, Barleben, Germany), this was stored at $4^{o}C$ for analysis after the test. Following a five minute warm up (5-10 $km.hr^{-1}$), participants commenced running at 2 km.hr below their predicted 10 mile pace (predicated from the most recent race the participant had completed). Each stage was 2 minutes in duration and the treadmill speed increased 1 $km.hr^{-1}$ per stage and incline remained 1% throughout. After each stage participants stopped for 45 seconds for a finger tip blood sample to be collected. Expired gas was analysed with an automatic gas analyser to establish the rate of oxygen consumption, the analyser was calibrated prior to each test. HR was recorded with a Polar HR monitor (Polar Electro Oy, Kempele, Finland) and rating of perceived exertion (RPE) on a 6-20 Borg scale was recorded in the final 15 seconds of each stage, participants continued running until volitional exhaustion. The average test length was 18 minutes.

Lactate Threshold and percentage VO_{2max}

Blood lactate measures were taken at rest and after each two minute stage in the VO_{2max} test and was measured with a BioSen C line (EFK Diagnostic, Barleben, Germany) machine. Lactate threshold was deemed to be 1 mmol/L above the resting value (Yoshida *et al*., 1987). Blood lactate values were plotted against speed and speed at lactate threshold determined from the resulting graph. Percentage VO_{2max} at lactate threshold was calculated by plotting speed against VO2 and interpolating the resultant regression equation.

Familiarisation

During the VO_{2max} test participants were given an opportunity to familiarise themselves with the changing speed of the treadmill which would occur during the interval session. After the VO_{2max} test participants were asked to produce a saliva sample to familiarise themselves with the collection procedure. Instructions were also given about the circuit

exercises to be completed before participants began this trial, and time was allocated to practice the exercises with guidance if required.

6.2.3 Main trials

In visits 2-5, participants undertook four main trials, there were: (a) circuit session; (b) interval session; (c) tempo run or (d) rest trial, as described below. They were completed in a randomised order on separate visits to the lab with at least three days between each trial.

Circuit session

Participants completed three sets of ten exercises (figure 7.01). These exercises were performed on a mat where necessary, while standing on the floor, in the case of the tricep dips these were performed on a box (30 cm) and the step ups on a bench (35 cm). Each exercise was performed for 30 seconds with 30 seconds recovery between exercises and began with sit-ups. Heart rate was recorded every 1.5 minutes and RPE every three minutes during the exercise. This session aimed to simulate an endurance circuit session to improve core strength.

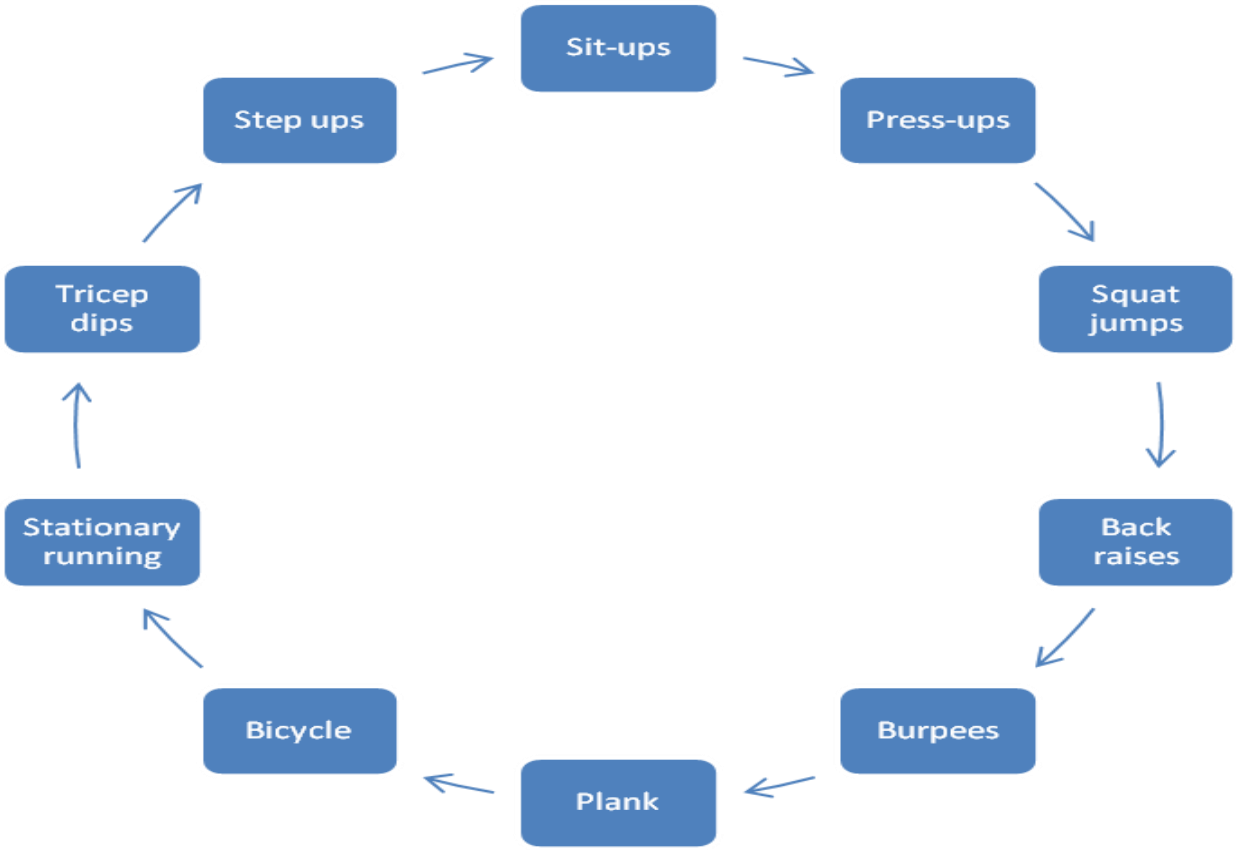


Figure 6.01 Circuit session exercises

Tempo run

The tempo run was performed for 30 minutes at a constant speed which coincided with lactate threshold after the VO_{2max} test. Heart rate was recorded every 1.5 minutes during the trial and RPE every three minutes.

Interval session

This session lasted 31 minutes in duration and was divided into intervals and recovery period. Intervals were 3.5 minutes in duration at a treadmill speed equivalent to speed at 90% VO_{2max}. Recovery periods were two minutes duration at a velocity equivalent to 30% VO_{2max}. Participants completed six repetitions during the trial. Heart rate was recorded every 1.5 minutes and RPE every three minutes. Heart rate was then adjusted for the proportion of time spent during recovery (32.3%) and repetitions (67.7%).

Rest trial

Participants sat and rested for the 30 minutes duration of this trial. HR was measured every three minutes.

6.2.4 Blood sample collection

Prior to the interval session, tempo run and rest trial participants were fitted with a cannula in the forearm (21G Venflon, Becton, Dickinson and Co., Oxford, United Kingdom). In any case where it was not possible to cannulate the participant, serial blood samples were taken by venepuncture (21G BD Vacutainer Safety-Lok blood collection set; Becton, Dickinson and Co.) from an antecubital vein and this procedure was then used for all trials. During and after the circuit session all samples were taken with the aforementioned butterfly needles as there was a risk the cannula could be dislodged during the activities or when the participants were moving between them. Blood samples were collected into 6 mL tripotassium ethylenediaminetetraacetic acid (K_3EDTA) Vacutainers (Becton, Dickinson and Co.) pre exercise and 0, 15, 30 and 60 mins post exercise. 2 mL stimulated saliva samples were also taken at these time points (using the collection procedure mentioned previously). Blood samples were refrigerated at 4^{o}C (for no more than two hours) until the end of each trial. Samples were identified as being stable for up to four hours at 4^{o}C prior to centrifugation and freezing (Tuck *et al., 2*008). After each trial blood samples were centrifuged at 1500 x G for 10 minutes, plasma was then aliquoted into four labelled Eppendorf tubes and stored at -80 ^{o}C until analysis.

6.2.5 Saliva sample collection

Saliva samples were collected at the pre exercise, and 0, 15, 30 and 60 minutes post exercise. The saliva collection was based on the method described in Chapter 2. Participants could drink water ad libitum during all the trials but were advised to stop drinking 5 minutes before each saliva sample to avoid dilution. Samples were refrigerated at 4^{o}C until after the trial ended and were then processed and stored.

6.2.6 Sample analysis

ELISA

Saliva was analysed for cortisol and testosterone with ELISA as detailed in Chapter 2. The mean intra assay coefficients of variation were 8.0% for cortisol and 9.1% testosterone for duplicate samples. The mean inter assay coefficients of variation were 7.4% and 5.2 % for cortisol and testosterone, respectively.

Plasma cortisol and testosterone concentrations were determined using commercially available ELISA kits (DRG Instruments, Germany). All samples were thawed and inverted three times prior to analysis. Plasma (25 µL) was measured in duplicate according to the kit instructions. The sensitivity of the kits were 2.5 ng/mL for plasma cortisol and 0.083 ng/mL for plasma testosterone. The mean intra assay coefficients of variation were 9.3% for cortisol and 6.1% for testosterone. The mean inter assay coefficients of variation were 6.2% and 7.5% for cortisol and testosterone, respectively.

LC-MS

Saliva samples (1 mL) collected before and after the INT and TEMP sessions were also analysed for cortisol using LC-MS. They were centrifuged and stored in the same way as the ELISA samples. The method used was optimised and validated in previous chapters and is detailed in chapter two.

6.2.7 Statistical analysis

A one way ANOVA with repeated measures design was used to examine HR and RPE data. Data was checked for normality prior to testing and those data not eliciting normal distribution were log transformed prior to analysis (salivary cortisol and testosterone and C/T data). A two way ANOVA (4 trials x 5 sample points) with repeated measures design

was used to examine the salivary data. Significant differences were assessed with student t-test with Bonferoni post hoc adjustments for multiple comparisons. Pearson's product moment correlation coefficient and agreement analysis were used to assess correlations between salivary and plasma measures and ELISA and LC-MS data. Some graphs are presented as ± standard error of the mean (SEM) for clarity due to large SD. Statistical significance was accepted at $P < 0.05$.

6.3 Results

6.3.1 Trial characteristics

The characteristics in terms of running speed, percentage VO_{2max} and percentage maximal HR for each trial are presented in table 6.2. Percentage maximum HR was significantly higher in INT and TEMP compared to CIR (main effect of trial, $F_{2, 12} = 84.4$, $p<0.0001$) but did not significantly differ between INT and TEMP trials.

Table 6.2 Mean ± SD for treadmill speed and VO_{2max} for tempo and interval session trials (n=10)

Trial (mean ± SD)	Treadmill Speed ($km.h^{-1}$)	VO_{2max} (%)	Maximum HR (%)
Tempo	13.0 ± 1.4	74.7 ± 1.6	87.1 ± 6.1*
Intervals - repetition	15.3 ± 1.6	88.3 ± 3.2	
Intervals - recovery	3.6 ± 0.7	30.6 ± 3.3	
Intervals - mean	11.6 ± 1.3	66.5 ± 3.0	86.0 ± 7.1*
Circuits	n/a	n/a	67.4 ± 7.5

*significantly higher than CIR ($P<0.05$)

6.3.2 HR and RPE

Mean HR increased with the exercise sessions and was greater in INT and TEMP compared with CIR (main effect of trial $F_{3, 21} = 318.9$, $p<0.0001$). However, subsequent post hoc tests revealed no difference was observed between INT and TEMP. There was also a significant effect of trial for peak HR ($F_{2, 16} = 216.7$, $p<0.0001$), Bonferoni pairwise comparisons revealed peak HR was significantly higher in INT compared to CIR and TEMP ($p<0.05$) (table 7.3). RPE was significantly different between trials (main effect of trial ($F_{2, 16} = 8{,}809$, $p<0.01$). Further analysis revealed RPE was higher for INT compared to TEMP ($p=0.004$) and CIR ($p=0.021$)

Table 6.3 Mean and Peak (HR ± SD) (b.p.m) and mean RPE for all trials (n=10).

b.p.m (± SD)	Rest	TEMP	INT	CIR
Mean HR	59 ± 6	155 ± 10*#	145 ± 12*#	116 ± 10*
Peak HR	N/A	163 ± 10	173 ± 12§	148 ±10
RPE	6.0 ± 0	14 ± 2	15 ± 2§	13 ± 1

*significantly higher than rest (P<0.01), # significantly higher than CIR (P<0.01), § significantly higher than CIR and TEMP (P<0.01)

The heart rate response varied between trials (figure 6.02). TEMP showing a gradual significant rise in HR throughout the duration of the trial (135 ± 15 rising to 159 ± 11, p=0.001). However, in the INT session there was a significant increase in HR during the 3.5 min compared to the recovery periods between repetitions (160 ± 11 bpm versus 114 ± 12 bpm, p=0.0001). CIR showed intermittent increases and decreases in HR during the trial, however HR never exceeded TEMP.

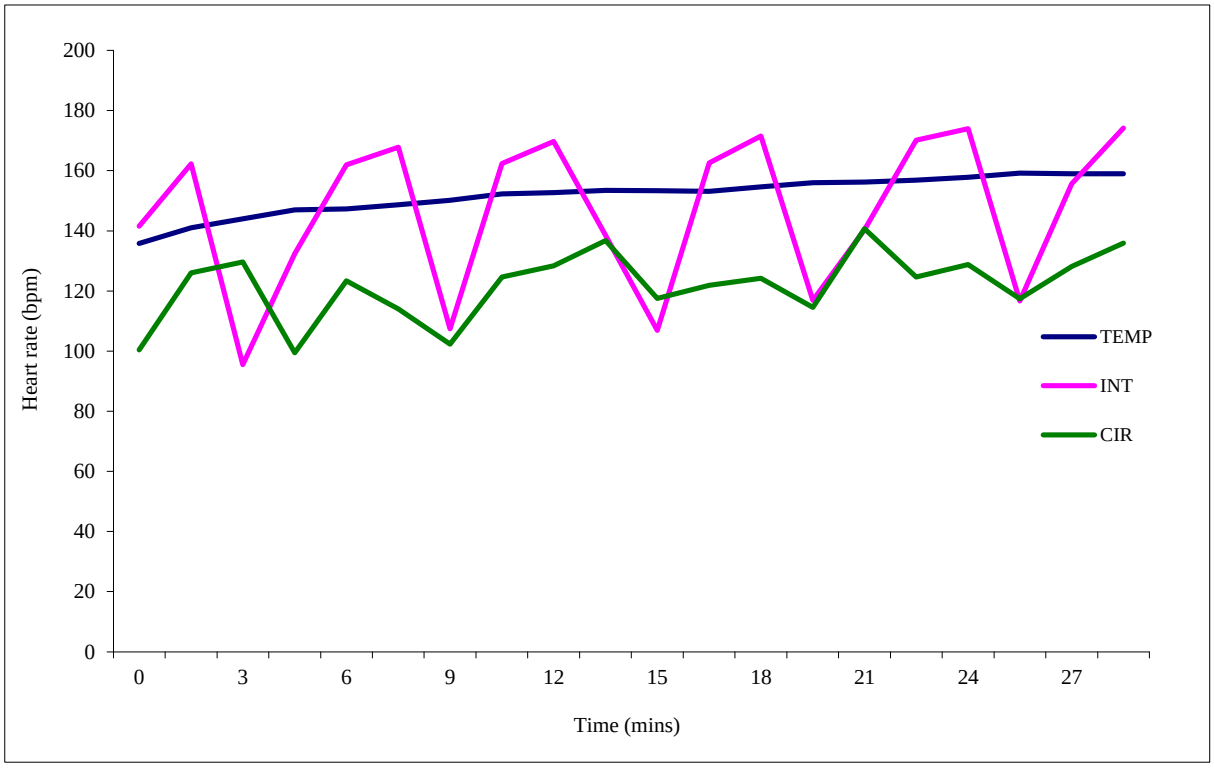


Figure 6.02 Mean HR for INT, TEMP and CIR across time (n=8)

6.3.3 Salivary cortisol response

Salivary cortisol response for the four trials is presented in figure 6.03. There was no change in cortisol levels for the duration of the resting trial. There was a trend for post exercise salivary cortisol levels to be higher than pre exercise (p=0.064) and 15 mins recovery (p=0.068). Salivary cortisol concentration was also significantly higher in INT compared to rest (p=0.004) and TEMP (p=0.046). There was a trial x time interaction ($F_{12,84}$= 5.4, p<0.0001) and INT session elicited significantly higher salivary cortisol levels at all recovery sample points compared to pre exercise.

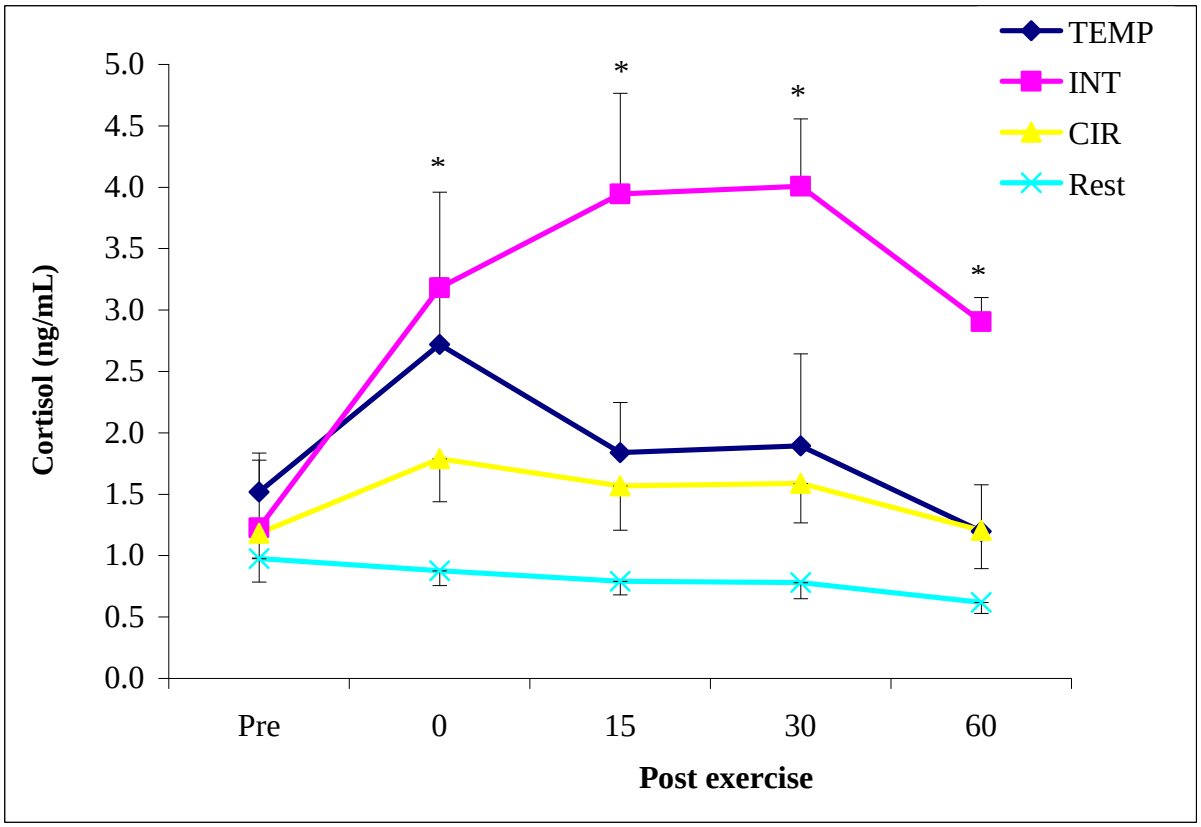


Figure 6.03 Mean values (+/- S.E.M) for cortisol in response to rest, TEMP, INT and CIR sessions (n=10) *significantly higher than pre exercise (p<0.05)

AUC analysis (table 6.4) revealed differences in the cortisol response (main effect of trial, $F_{3, 24}$ = 8.20, p<0.001). Bonferoni post hoc tests showed INT trial elicited significantly higher cortisol response compared to rest (p=0.025) and CIR (p=0.047) but not TEMP.

Table 6.4 Mean (± SD) salivary AUC for cortisol and testosterone for rest, INT, TEMP and CIR and plasma INT and TEMP

AUC	Cortisol		Testosterone	
	Saliva	Plasma	Saliva	Plasma
Rest	70.4 ± 28.4		9352 ± 3698	
INT	282.1 ± 156.5*#	15503 ± 4577	13735 ± 3111*	456645 ± 116482
TEMP	172.0 ± 120.2	11324 ± 5365	13080 ± 3972*	511734 ± 220859
CIR	127.9 ± 81.6		12248 ± 3198	

*significantly higher than rest ($p<0.05$), # significantly higher than CIR ($p<0.05$)

6.3.4 Salivary testosterone response

There was no change in testosterone levels for the duration of the resting trial. However in the exercise trial there was a difference in salivary testosterone (main effect of trial $F_{3,24} = 6.305$, $p<0.003$). Subsequent Bonferoni comparisons revealed TEMP was significantly higher than rest ($p=0.05$) and INT showed a trend to be higher than rest ($p=0.061$) (Figure 6.04). There was also an effect of time ($F_{4, 32} = 17.04$, $p<0.0001$), with an increase in testosterone levels pre to immediately post exercise ($p=0.001$). Moreover, there was a significant reduction in testosterone concentration at all time points post exercise compared to 60 minutes recovery ($p<0.01$). A trial by time interaction ($F_{12,96} = 5.72$, $p=0.001$) revealed that TEMP ($p=0.021$), INT ($p=0.028$) and CIR ($p=0.0001$) were all significantly higher post exercise compared to pre exercise. An ANOVA comparing AUC between trials illustrated a difference (table 7.4) (main effect of trial, $F_{3, 24} = 6.93$, $p<0.002$) and post hoc tests found TEMP ($p=0.047$) and INT were significantly higher than rest ($p=0.043$).

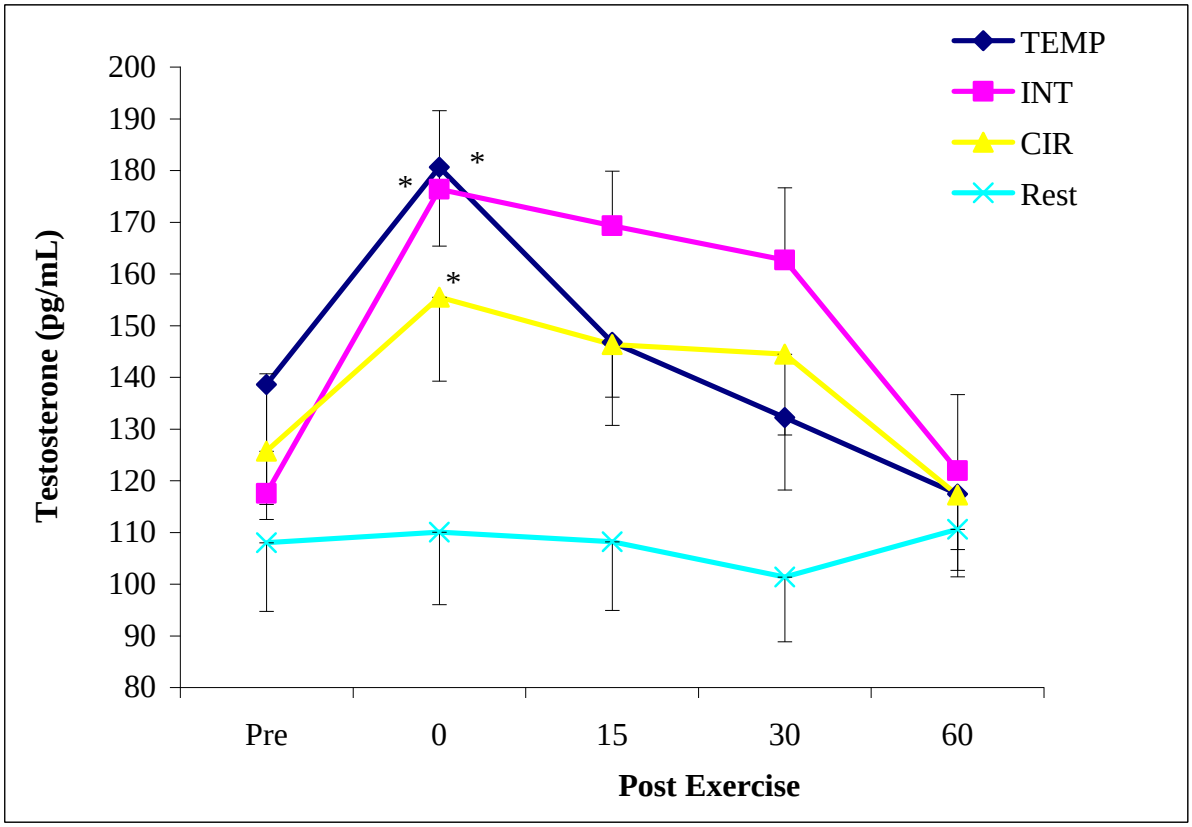


Figure 6.04 Mean values (+/- S.E.M) for testosterone in response to rest, TEMP, INT and CIR sessions (n=10). *significantly higher than pre exercise

6.3.5 Salivary cortisol/testosterone (C/T) ratio

An ANOVA revealed salivary C/T ratio differed between trials (main effect of trial, $F_{3, 21}$ = 8.20, p<0.001) and increased significantly after INT compared to rest (p=0.009), however there was no difference between any other trials (figure 6.05). A trial x time interaction ($F_{12, 84}$ = 4.49, p<0.001) followed by post hoc tests showed INT was significantly higher than pre exercise at all time points post exercise (p<0.05).

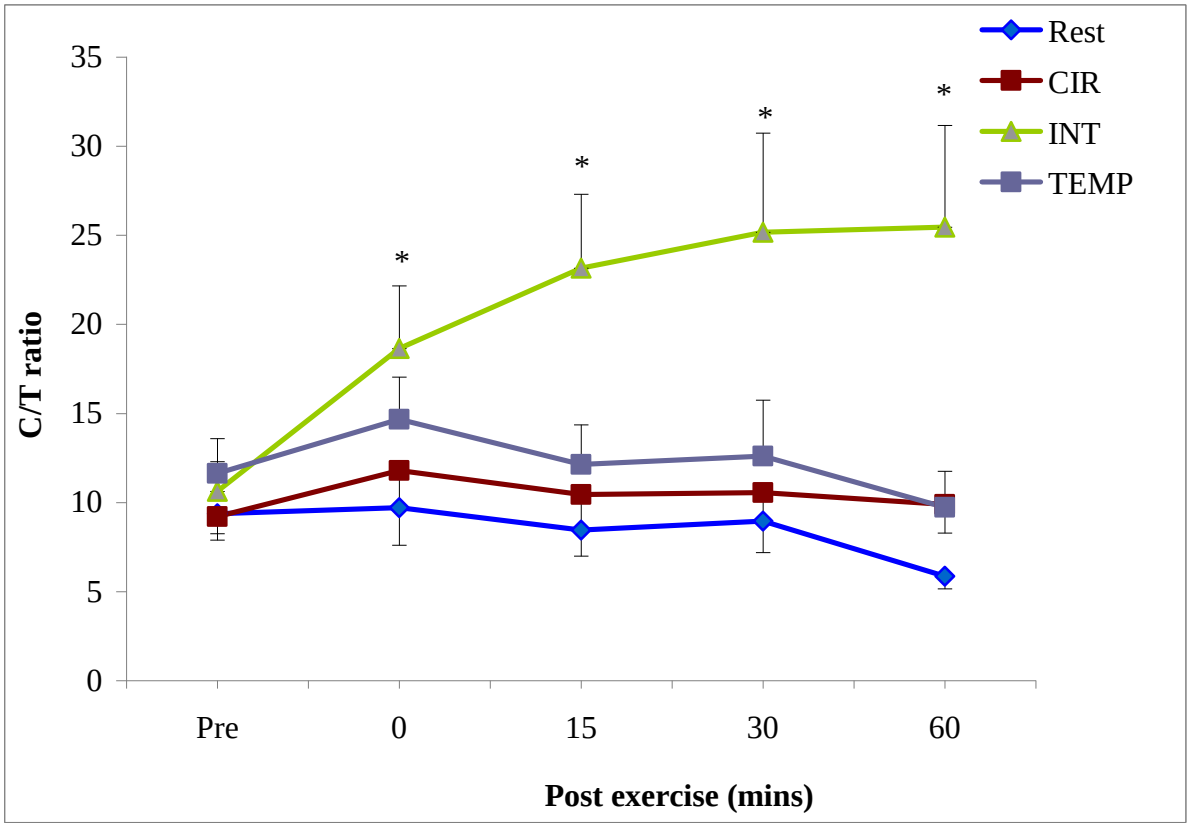


Figure 6.05 Mean values (+/- S.E.M) for salivary C/T ratio in response to rest, TEMP, INT and CIR sessions (n=10) *significantly higher than pre exercise (P<0.05)

6.3.6 Plasma and salivary hormone correlations

Levels of cortisol and testosterone in plasma were analysed from TEMP and INT sessions and results used to correlate against salivary measures. Plasma showed a higher mean cortisol concentration compared to saliva (145.3 ± 68.0 ng/mL vs. 2.41 ± 1.89 ng/mL respectively). This trend was mirrored in salivary (145.7 ± 48.1 pg/mL) and plasma (5518.9 ±1873.0 pg/mL) testosterone ($p<0.0001$). Plasma cortisol values for both trials compared to saliva are presented in figure 6.06 and testosterone in figure 6.07.

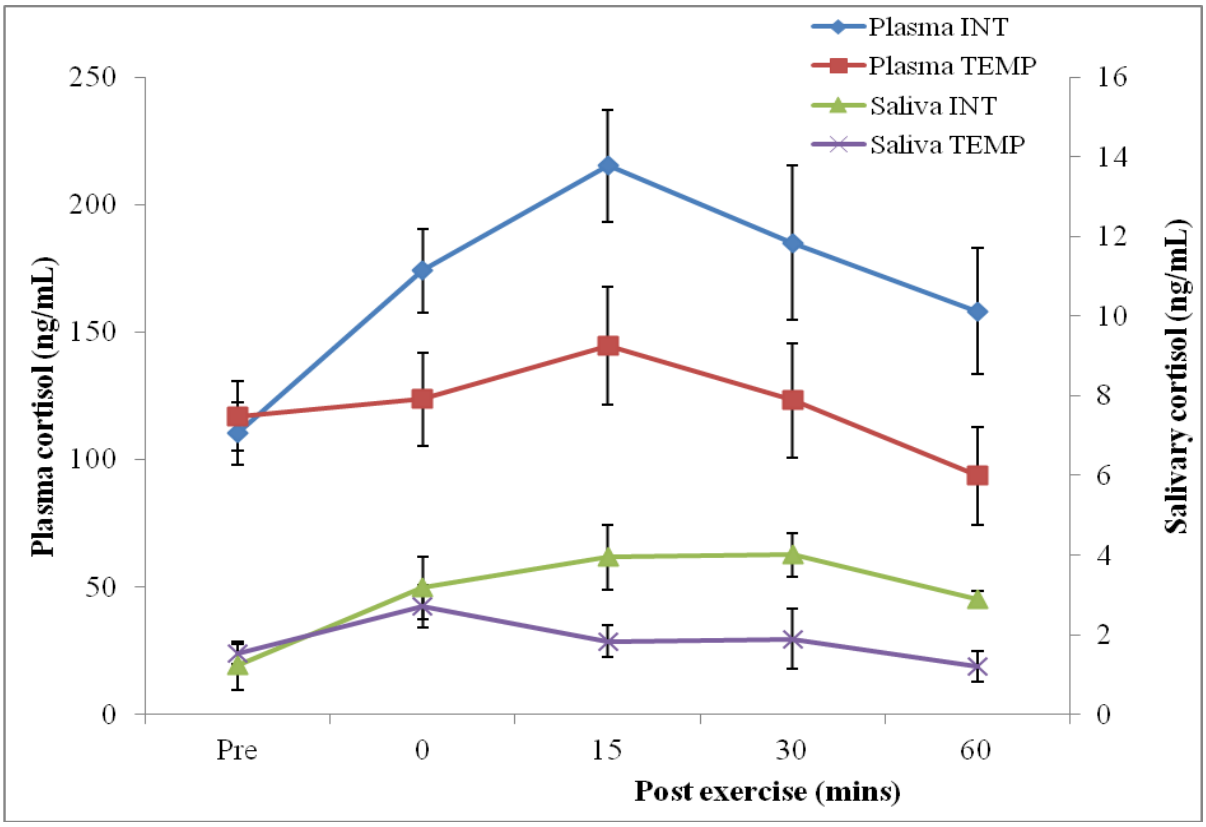


Figure 6.06 Mean (± SEM) salivary vs plasma cortisol for tempo and interval session (n=10).

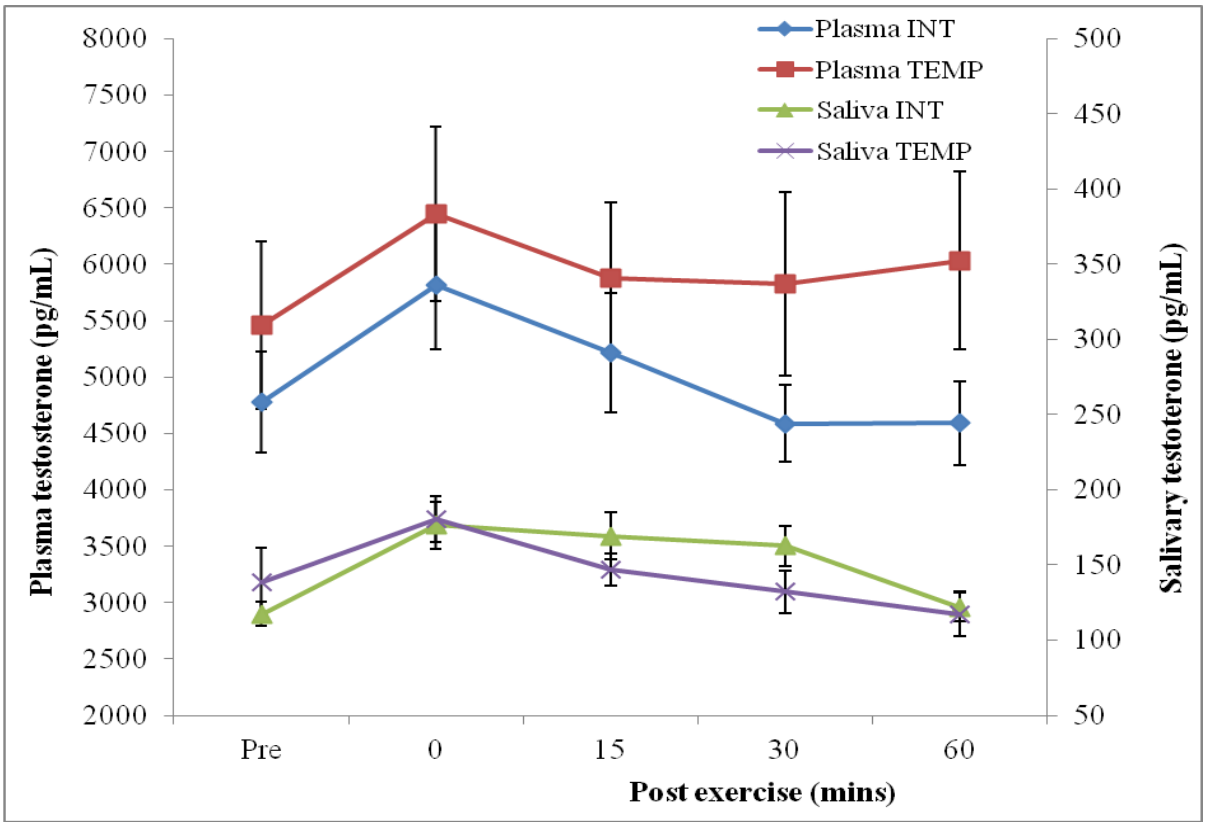


Figure 6.07 Mean (± SEM) salivary vs plasma testosterone for tempo and interval session (n=10)

Plasma and salivary measures from all time points in TEMP and INT sessions were also compared to establish if there was a correlation (table 6.5). Overall there was a correlation between saliva and plasma cortisol (r=0.813, p=0.0001) (figure 6.08) and testosterone levels (r=0.568, p=0.0001) (figure 6.09).

Table 6.5 Correlation between salivary and plasma cortisol and testosterone levels for tempo and interval sessions (n=10)

	Cortisol	Testosterone
TEMP	r=0.816, p=0.0001	r=0.671, p=0.0001
INT	r=0.529, p=0.0001	r=0.479, p=0.001

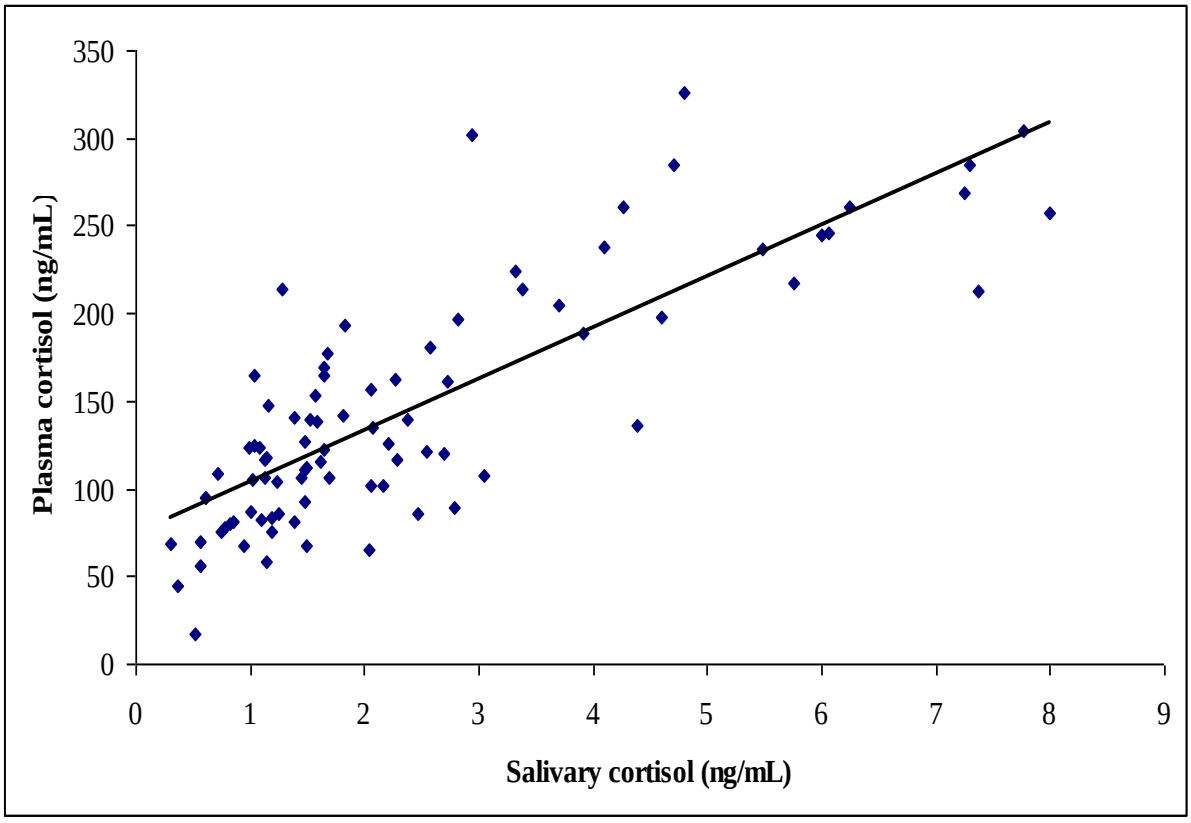


Figure 6.08 Correlation between plasma and salivary cortisol (n=87)

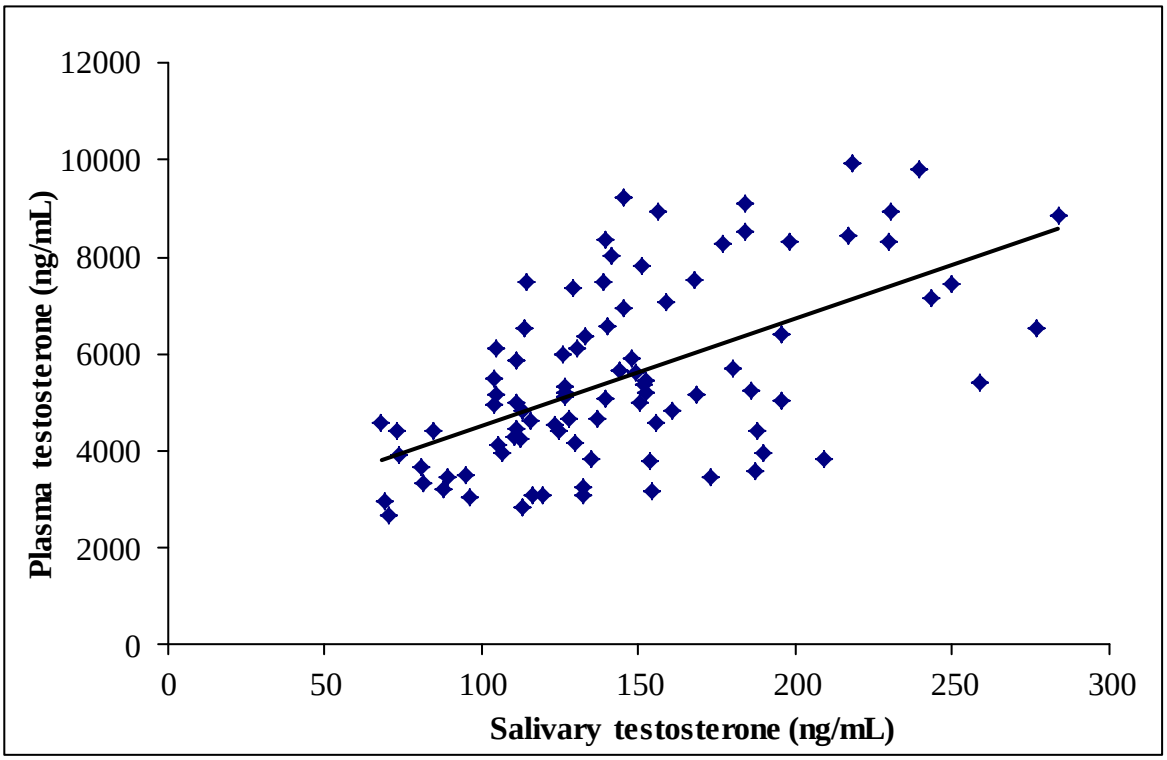


Figure 6.09 Correlation between plasma and salivary testosterone (n=91)

Comparison of peak hormonal measures revealed post TEMP salivary cortisol peaked immediately after exercise and plasma levels 15 minutes post exercise (figure 6.04). There was a correlation between peak post exercise plasma and salivary cortisol levels for this session (r=0.887, p=0.001). Salivary testosterone measures peaked immediately after TEMP and plasma levels also peaked at this time and exhibited a correlation between peak post exercise salivary and plasma hormone concentration (r=0.792, p=0.011).

After INT plasma and salivary cortisol levels peaked at 15 mins post exercise and testosterone levels immediately post exercise. There was also no correlation between peak post exercise salivary and plasma measures for either hormone after INT.

6.3.7 Method validation

Salivary cortisol samples (INT and TEMP) were compared with ELISA and MS (figure 6.10). This revealed a strong correlation between the techniques (r=0.934, p=0.0001). Additionally, comparison between MS and ELISA samples for INT revealed no significant difference between the trials (figure 6.11), although MS samples showed a trend towards being lower than ELISA (p=0.099). Salivary testosterone was also examined with LC-MS

and there was no correlation with ELISA (figure 6.13; Appendix 5). Limits of agreement analysis for cortisol revealed the agreement between MS and ELISA was 0.83 ng/ml below and 1.19 ng/ml above (figure 6.12).

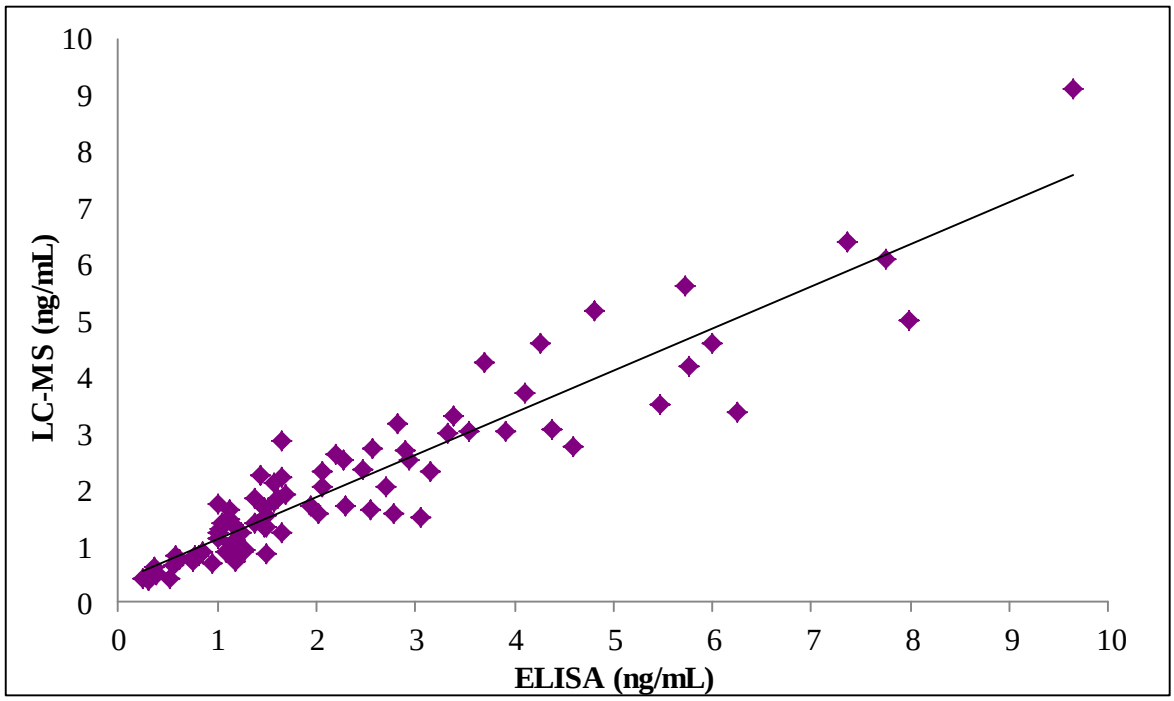


Figure 6.10 ELISA vs. MS for salivary cortisol exercise samples (n=85)

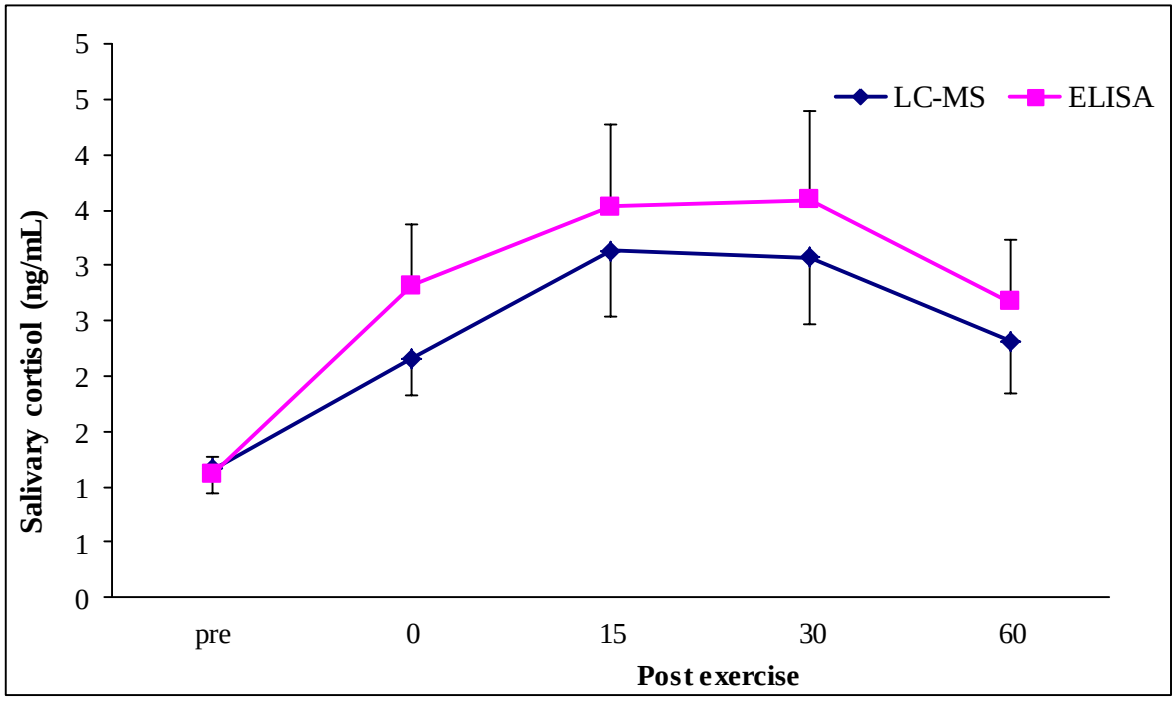


Figure 6.11 Mean (± SD) for salivary cortisol measured before and after INT with LC-MS and ELISA (n=8)

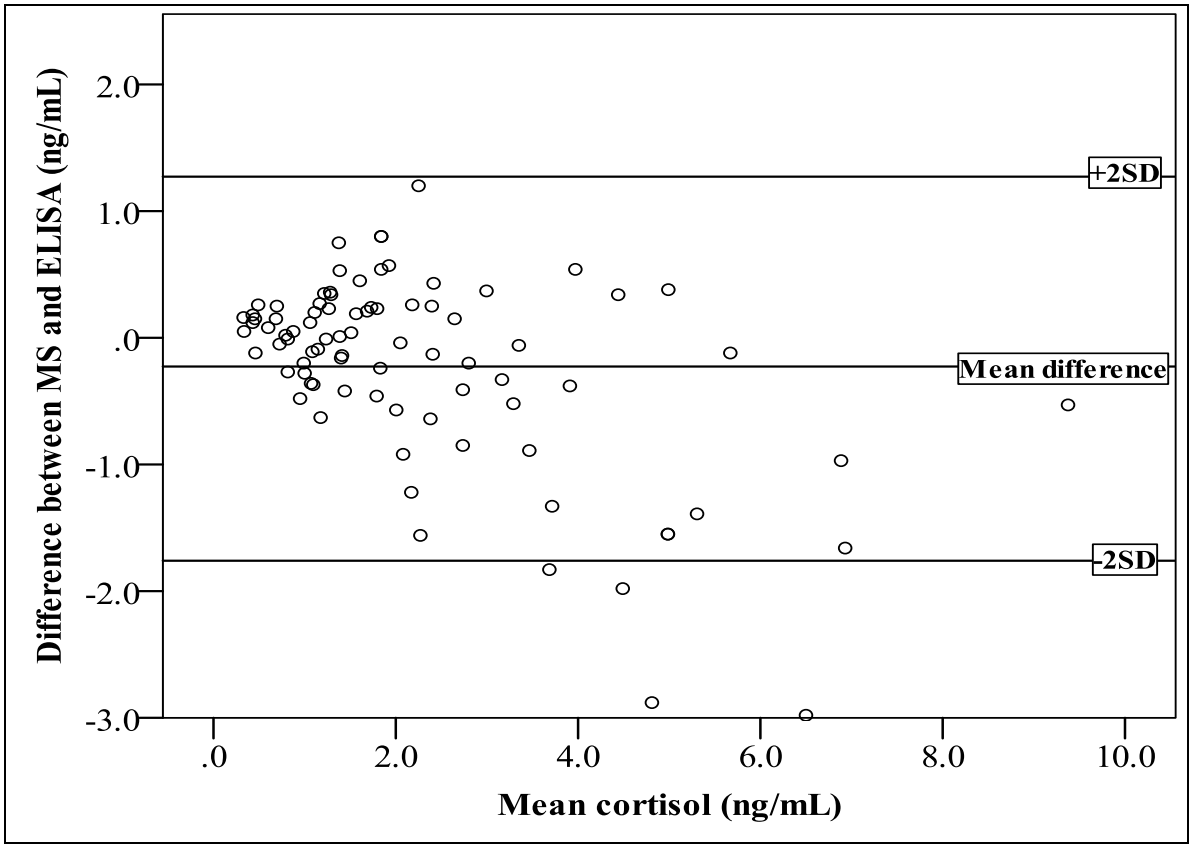


Figure 6.12 Upper and lower agreement limits (+/-2SD) to compare MS and ELISA assays (n=85)

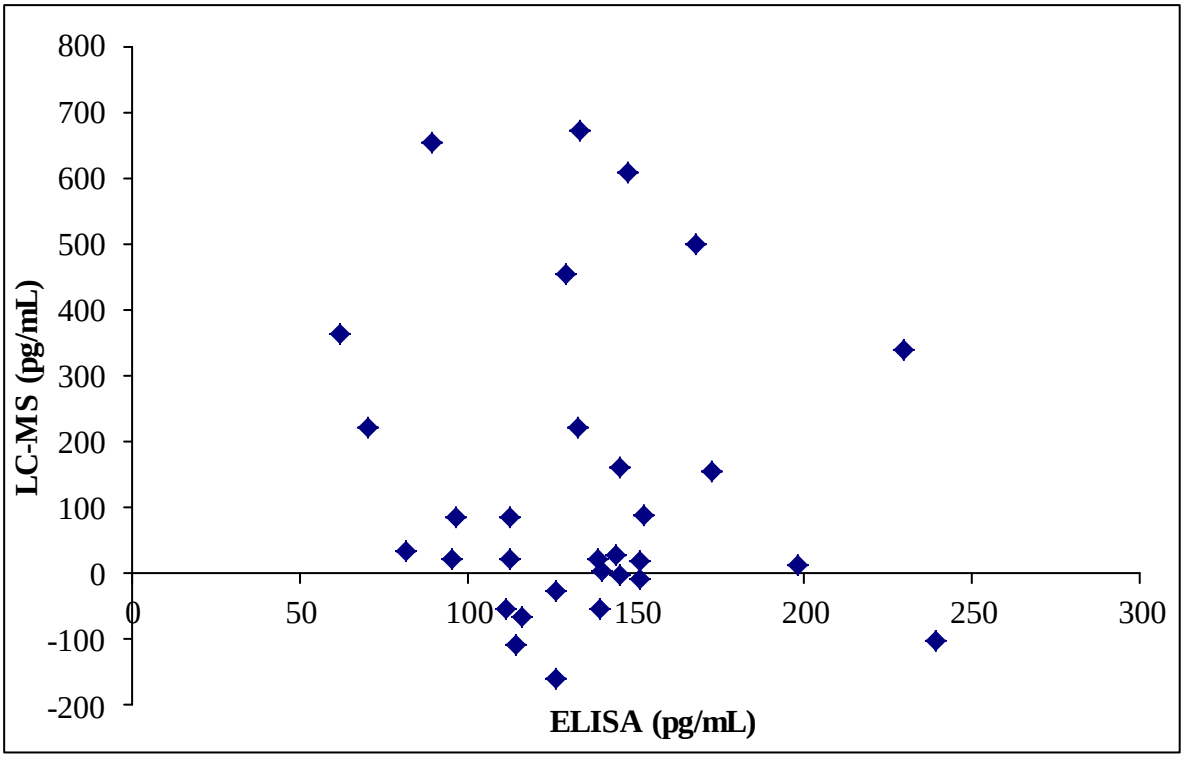


Figure 6.13 Comparison between ELISA and LC-MS for quantification of testosterone (n=31)

6.4 Discussion

The main findings of the present study were: 1) an INT session resulted in a prolonged increase in salivary cortisol levels post exercise; 2) salivary testosterone levels increased in response to exercise; 3) salivary C/T ratio increased in response to INT; 4) there was a correlation between salivary and plasma cortisol and testosterone concentrations; 5) Peak post exercise salivary and plasma cortisol and testosterone concentrations varied with exercise mode; 6) LC-MS is a valid tool to measure salivary cortisol.

A significant increase in salivary cortisol concentration was observed after the INT session compared to rest and this was also significantly higher than TEMP. The nature of the exercise and RPE response may help to explain these findings. Participants reported the INT session to be more strenuous overall with a significantly higher RPE. This may reflect the periods of high exertion during the INT session, participants reached 96.1% of maximum HR during the 3.5 min repetitions, and a significant increase in HR was observed compared to the recovery periods. There was no difference in mean HR between INT and TEMP, although peak HR was significantly higher in the INT trial compared to TEMP. The increase in salivary cortisol response after INT and prolonged high levels observed at all recovery time points may reflect the higher metabolic demands and activation of the HPA axis leading to cortisol secretion during this trial. Despite significantly higher cortisol levels at 60 minutes of recovery post INT, it is likely that levels would have returned to baseline given further recovery. Typically prolonged endocrine disturbances are only observed after extremely intense exercise such as intensive training or excessively prolonged exercise over hours or days (Hackney and Viru, 1999).

TEMP showed a gradual rise in HR throughout the duration of the trial however the demands of this trial were not sufficient to influence a significant increase in salivary cortisol. Studies have shown a gradual increase in cortisol levels with exercise duration, this may be due to increased production and reduced metabolic clearance (McMurray and Hackney, 2000). Therefore increasing the duration of the TEMP trial would be likely to elicit a significant increase.

Other studies examining the cortisol response to exercise have found mixed results; the present findings are supported by Hough *et al.,* (2011) who after a cycling interval session

(30-minute continuous cycle alternating 1 minute at 55% work rate max and 4 minutes at 80% work rate max) reported a prolonged increase in cortisol levels above baseline after 60 mins recovery. However the present results differed to those reported by Vuorimaa *et al.,* (2008). They tested a 40 minute tempo run at 80% velocity of VO_{2max} and a 40 minutes repetition session which consisted of two minutes run (100%vVO_{2max}) and two minutes recovery (slow walk). Both trials showed a significant increase in serum cortisol concentration post exercise although the increase 10 minutes post tempo run was higher than that observed for the interval session. The reason for the differences may have been due to the strain of the protocols, as Vuorimaa and colleagues report the total time but not the total work output of tempo and intervals runs were equated, and this resulted in similar RPE measured at the end of the 40-min exercises. They suggested that if the total work output was equal, the serum cortisol response to the intervals session may have been greater than tempo. The present study reports a higher RPE in INT and this may reflect the higher work output of this trial. Furthermore it is recognised that cortisol has anti-inflammatory properties (Brook and Marshall, 2001) and perhaps muscle damage during the high intensity interval session may have elicited an increased cortisol response as a protective measure.

An intensity threshold has been proposed to elicit increases in cortisol levels (Allgrove *et al.,* 2008; Budde *et al.,* 2010; O'Connor and Corrigan, 1987; Rudolph and McCauley, 1998; Hough *et al.,* 2011). The present study included exercise above 60% VO_{2max} for 21 mins in INT and 30 mins in TEMP. Results from the INT session supports the consensus that exercising above 60% VO_{2max} for >20 mins will elicit a significant increase in cortisol. However, the present study refutes this hypothesis as there was no significant increase in cortisol levels after the TEMP trial despite meeting the threshold criteria.

Salivary testosterone levels also increased in response to exercise. The present study observed an increase in testosterone post exercise after INT, TEMP and CIR. These findings are supported by Vuorimaa *et al*., (2008) who found similar increases in serum testosterone after INT and TEMP running trials. Hough *et al.,* (2011) also showed an increase in testosterone levels after two cycling interval sessions with testosterone returning to baseline within one hour of cessation of exercise, and Kokalas *et al.,* (2004) showed in increase after a rowing interval session. Various mechanisms have been proposed for this exercise induced increase. It has been suggested that dependance on the

intensity and duration of the exercise is caused by the influence of increased circulation in the testes, activation of the sympathetic nervous system, increased lactate accumulation and/or lutenising hormone (LH) concentrations (Eik-Nes, 1969; Jezova & Vigas, 1981; Fahrner & Hackney, 1998). Acute exercise induced changes can be caused either by the testicular level (Leydig cells) or by the hypothalamic–pituitary level. In their study, Vuorimaa *et al.,* (2008) also measured LH and follicle stimulating hormone (FSH) and observed changes in testosterone but not LH or LSH. Therefore they suggest the changes in testosterone are caused by the testicular level and not the hypothalamus–pituitary level. This is refuted by Vasankari *et al*., (1993) who demonstrated that an acute exercise-induced decrease in testosterone was caused by reduced hypothalamus-pituitary stimuli. There is also suggestion that circulating catecholamines may influence testosterone levels (Jezova & Vigas, 1981) however further research is required to examine this mechanism. Dissimilarly to cortisol salivary testosterone returned to baseline after INT which suggests that there may be a different mechanisms for release occurring after exercise stress in these hormones. This finding supports the hypothesis that most exercise induced testosterone secretion is from the testes and not the hypothalamus (Vuorimaa *et al.,* 2008).

No previous studies have investigated the response of cortisol and testosterone to aerobic circuit training however mobilisation of testosterone after this trial may reflect reports observing increases in testosterone after resistance exercise (Yarrow *et al.,* 2007; Kraemer *et al.,* 1990; Raastad *et al.,* 2000; Hakkinen *et al.,* 1993, Linnamo *et al.,* 2005). There is no clear trend regarding cortisol response to resistance exercise and the present study adds to findings that observed no change in cortisol levels after weight training (Fry and Lohnes, 2010). The circuit training session contained a combination of weight bearing and aerobic exercises and further research is required to clarify the mechanisms of testosterone release and relate this to other running training sessions.

Examining the salivary C/T ratio revealed only INT showed an increase and this was observed at all time points post exercise. The increase is likely to reflect the cortisol response to exercise as a similar trend was observed in this measure. It is apparent that salivary testosterone levels did not increase sufficiently to influence the C/T ratio. The increase in cortisol and testosterone secretion after INT may be due to the adrenal glands secreting cortisol and testosterone concurrently (Kroboth *et al.,* 1999). These hormones are also formed from the same precursor, and an increased concentration of cortisol in

circulation may cause some dissociation of free testosterone from its carrier proteins, as the two hormones compete for binding sites (Rosner, 1990). Despite the proposed mechanisms further investigation is required to clarify the mechanism (Brownlee *et al.,* 2005). Few studies have reported the C/T ratio in response to acute exercise and its use in this context is limited. There is however increasing evidence that the C/T ratio may be a useful tool to monitor chronic training and recovery status (Argus *et al.,* 2009). Under certain circumstances a negative relationship exists between cortisol and testosterone representing a shift to catabolism, which can have a negative effect on training and performance (Urhausen *et al*., 1995). Cumming *et al.,* (1983) found that using pharmacological doses of cortisol induced a decrease in testosterone production. However the strength of this in vivo relationship in response to exercise has not been thoroughly determined. It has been postulated that a decrease in testosterone may be due to inhibition of production in the testes (Cumming *et al.,* 1983) and potentially binding of LH on the testes and steroidogenesis (Bambino and Hsueh, 1981; Castro and Matt, 1997).

Plasma and salivary cortisol and testosterone levels elicited a correlation in the present study. There was a higher correlation between blood and saliva in TEMP compared to INT. This reflects the inter participant variation in cortisol and testosterone response to INT. Other studies have revealed a correlation between blood and salivary measures. Hough *et al* (2011) reported a higher correlation between peak salivary and plasma cortisol levels in a run to fatigue compared to two INT trials. Similarly, O'Connor and Corrigan (1987) revealed a strong correlation between serum and salivary cortisol measures during and after 30 minutes of cycling at 75% VO_{2max}. Results from the present study confirm the validity of salivary measures when monitoring the cortisol and testosterone response to short term intermittent and continuous exercise. Salivary measurements are beneficial as a less invasive technique than blood measurement. Saliva is particularly useful to examine the stress response as stress caused by blood taking can be avoided. Salivary measurement also allows participants to collect their own samples with minimal instruction and samples can be posted to the laboratory if necessary.

Results also revealed variation in post exercise hormone peaks. Plasma and salivary cortisol and testosterone levels peaked simultaneously in all trials. This refutes the hypothesis of a post exercise delay in peak cortisol and testosterone in saliva compared to blood. Studies have reported this delay for resting cortisol levels (Kirshbaum and

Hellhammer, 1989; Henrichs *et al.,* 2001) and after exercise (O'Connor and Corrigan, 1987; Crewther *et al.,* 2006; Hough *et al., 2*011; Daly *et al., 2*005) showing delays of up to 30 mins for peak cortisol levels in saliva. The present results reflect support observations of an immediate diffusion from blood into saliva after intravenous injection (Kirschbaum and Hellhammer, 2000; Wang *et al., 1*981). The reason for the difference in the current trials may be that in previous studies vasoconstriction occurred in the arterioles that supply the salivary glands, resulting in lower salivary volume (Chicharro *et al*., 1998). The reduced blood flow may in turn explain the delay in cortisol and testosterone delivery and diffusion from blood into the saliva found after acute exercise. It may be that the exercise in the present study was of insufficient intensity or duration to elicit this change. Testosterone levels have also been found to correlate with blood at rest (Vittek *et al., 1*985; Wang *et al., 1*981). There was a variation in salivary cortisol levels between trials with a large inter individual difference in time for cortisol to peak post INT (immediately after to 30 minutes post exercise). The individual differences may have been caused by the intermittent nature of the trial. Other studies examining the time for hormones to peak post exercise have shown peak testosterone levels occurs earlier than cortisol within 10 minutes of cessation of exercise (Hough *et al., 2*011; Daly *et al., 2*005). However, the present study revealed cortisol and testosterone levels peaked immediately post exercise in all trials, with the only exception being the INT trial where cortisol levels peaked at 15 mins post exercise. The latter finding suggests during intense exercise the site of cortisol release may be different to testosterone, exemplified by the difference in peak hormone behaviour. The present study was conducted in running which involves weight bearing exercise with activation of large muscle groups. Most other studies reporting the delay in hormones have involved cycling exercise (O'Connor and Corrigan, 1987; Crewther *et al., 2*006; Hough *et al., 2*011) and running (Daly *et al., 2*005).

Validity of an LC-MS method to measure salivary cortisol and testosterone levels was also examined in the current study. Results showed there was a strong correlation between the salivary cortisol response to an INT and TEMP running session measured with LC-MS and ELISA. However testosterone could not be quantified with this technique, affirmed with the lack of correlation between LC-MS and ELISA. LC-MS concentrations tended to be lower than ELISA, this is suggested to be due to the lower specificity of ELISA leading to overestimation (Taieb *et al.*, 2003). The latter finding reflects the LOQ of the method for testosterone which was outside the reference range for male saliva samples. Testosterone

is harder to measure than cortisol due to lower levels present in saliva and its lower proton affinity which affects ionisation and detection with LC-MS. A downside of LC-MS is the high cost of instrumentation and time to develop assays can be restrictive, however once installed it allows single samples to be analysed immediately with simple cleanup which can be automated. Despite accurately representing the cortisol response to exercise, the LC-MS method is currently more time consuming than ELISA. Automation of cleaning procedures and use of UPLC would ensure this method is viable alternative to measure cortisol concentrations compared with ELISA. Additionally, multiple analyte testing to quantify salivary cortisol and testosterone levels in our laboratory still requires further development to offer a worthwhile alternative to ELISA testing.

In conclusion, these results suggest that in running sessions cortisol concentration is a useful indicator of acute exercise stress. Additionally the results negate the current proposed theory of a threshold intensity of cortisol and testosterone release. Salivary and plasma hormone levels were correlated in response to acute exercise; this supports the use of saliva as an alternative sampling technique to blood measurement of cortisol and testosterone levels. Additionally, the results partially refute the hypothesis of a post exercise delay in peak hormonal response to exercise and lag time for the hormonal response in blood and saliva. Finally, LC-MS represents an accurate tool to measure saliva cortisol levels in saliva and further work will cement its position as an alternative to ELISA.

7. GENERAL DISCUSSION AND CONCLUSIONS

7.1 Discussion

This research project gives an overview of the use of mass spectrometry as a tool for quantification of hormones such as cortisol and testosterone. The stages involved in development and validation of an LC-MS method for measurement of cortisol and testosterone levels in saliva are discussed and application to exercise are illustrated. This research aimed to validate an LC-MS method with resting saliva samples, and to evaluate the use of this method to quantify saliva samples collected from an exercise training study. The final aim was to investigate the hormonal response to different training sessions in runners, and assess the correlation of blood and salivary measures.

Chapter 3 showed that optimisation of MS and LC parameters are important to improve detection limits of cortisol and testosterone. Cone voltage and capillary voltage maintain a voltage gradient in order to form the Taylor cone during ESI and direct charged ions to the detector (Taylor, 1964). Additionally, if cone voltage is high, ions may have excess energy and collide leading to uncontrolled fragmentation of the pseudo molecular ion, this is confirmed by the increased production of insource fragments observed in this study. Ultimately fragmentation and low ion transfer can contribute to poor detection levels. A cone voltage of 25V and capillary voltage of 3.5 kV were identified as optimal in this method. During MS optimisation mobile phase solvent composition was also shown to influence analyte peak area. Evidence suggests that mobile phase composition can influence both MS and LC optimisation. Solvent choice and flow rate can also impact peak descriptives.. Solvent choice was examined and MeOH was chosen over MeCN. MeOH was chosen due to its protic nature, which promotes ionisation by readily giving a hydrogen ion for binding, potentially improving ionisation in less ionisable compounds and in turn increasing analyte signal. Additionally, there was a shortage of MeCN during method development and it was not cost effective to use this solvent (Tullo, 2008). Solvent percentage is also important; this was exemplified during MS optimisation where a 90% MeOH mobile phase elicited a higher peak area for cortisol and testosterone than 70% MeOH. Results suggest a higher percentage of organic solvent may elicit improved ionisation through more efficient evaporation and smaller droplets thus representing

improved transfer of ions into the gas phase (Smith *et al.*, 2002). Results also support the notion that more hydrophobic compounds compete better for charges during ESI compared to less hydrophobic molecules. Testosterone elicited a higher peak area than cortisol, which is in line with previous studies that showed more hydrophobic compounds elicit greater signal intensity (Null *et al.*, 2003). This is hypothesised to be due to a hydrophobicity effect during ESI (Marmur *et al.*, 2000). This concept has also been discussed by Abaye *et al*., (2011a; 2011b) whereby in mixtures; more non-polar analytes have a higher affinity for the ESI droplet-air interface, and the effective competition for the excess charge results in higher signal intensities in the mass spectrum. This phenomenon may be important to consider when examining biological matrices and sample preparation. Matrix effects common in biological samples have been identified to alter ionisation processes (Tang and Kebarle, 1993). Chapter 4 revealed the matrix of saliva contributed to a reduction in detection of testosterone by 6 %. Studies such as those presented above have postulated this is caused by variations in analyte polarity affecting the ionisation of different compounds through competition for charges. Additionally, interfering compounds may compete for these charges and suppress the signal of analyte of interest (Mallet, 2004). Therefore, it was concluded that appropriate cleaning (with SPE) and reconstitution of the sample should be untaken prior to analysis to further optimise detection and quantification.

It is also important to choose the most appropriate flow rate for the column (in this case 0.1 mL/min) and optimise the mobile phase composition to establish effective chromatography. In this study, a lower flow rate was shown to elicit higher peak area; this may be related to improved desolvation and transfer of ions with less mobile phase to evaporate during ESI (Gale and Smith, 1993). A narrow bore (2 mm) column was chosen as previous studies suggest smaller inlet diameter columns with low flow rates produce higher sensitivity than larger ID columns with higher flow rates (Shen *et al.*, 2002). This occurs due to the reduction in the cross-sectional area of the column, or square of the change in diameter which translates into proportionally taller peaks (Dolan, 2010). The gradient for the LC-MS method was optimised after consideration of the existing literature and the chosen ramped gradient method produced elution of tall, narrow peaks. Detecting analytes with different polarity such as cortisol and testosterone can benefit from a ramped mobile phase gradient as more polar compounds will be eluted from a column with a lower percentage of organic solvent than less polar analytes. Therefore, altering the percentage of

organic solvent during a chromatographic run allows optimisation of elution for both analytes. However the run time was long (15.5 mins) and this is inefficient for high throughput analysis and promotes the development of ways to speed up the chromatography for example utilising UPLC.

In order to establish accurate measurement of small analytes with LC-MS, peak resolution is also important in order to ensure specificity, accuracy and ultimately high S/N ratio. Examination of LM and HM parameters in the mass spectrometer revealed that although changes had minimal influence on peak area, optimisation was important to achieve mass accuracy and avoid merging of C12 and C13 isotopes, which can lead to an overestimation of peak area (Mallet *et al.,* 2004).

Column temperature has also been shown to have a strong influence on peak descriptives (VanHoenaker and Sandra, 2006; Bowermaser and McNair, 1984); although, the present study also refutes these observations with no difference in peak descriptives within the range of 25-50^{o}C tested. However only the column was heated and increasing the mobile phase temperature prior to column entry may have produced different results. Moreover, recent studies have shown availability of new columns able to withstand temperatures >200^{o}C, given the thermal stability of cortisol and testosterone these columns may be applicable to decrease analyte retention times and chromatographic run time; making the assay more viable for research applications. Mobile phase pH is a further factor which influences chromatographic performance, and decreasing the mobile phase pH had negative effects on peak area. 0.1% formic acid was optimal for the detection of cortisol and testosterone, increasing this percentage reduced the pH and consequently reduced peak area of cortisol and testosterone. When the percentage of buffer is increased, pH is lowered contributing to co-elution as the compounds of interest have minimal contact with the stationary phase of the column.

The main aim of this work was to develop a method to detect and quantify cortisol and testosterone levels in saliva, ionisation is an important factor impacting upon LC-MS detection limits especially regarding competition of charges. To apply an LC-MS method to analysis of saliva it was also important to consider the relevance of this matrix. Salivary measurement has many benefits over blood measures. Sample collection is less invasive therefore less likely to induce a stress response (Kraemer *et al.,* 2001). The sample can

also be collected by patients or study participants; therefore there is no need for medical personnel or researchers to be present. Additionally, plasma and salivary measures have been shown to be correlated. This is also observed in Chapter 6, when examining the plasma and salivary hormonal responses to exercise revealed plasma and salivary cortisol were strongly correlated. There was also a significant (but lower) correlation for testosterone. The present findings refute some previous exercise studies that have observed no correlation between plasma and salivary testosterone (Cadore *et al.,* 2008; Hough *et al.,* 2011); although the current findings are supported by correlations found at rest (Vittek *et al.,* 1985; Lac *et al.,* 1993). These results promote the use of saliva sampling to measure cortisol and testosterone in stress research.

However, due to the low concentration of cortisol and testosterone in saliva it was important to evaluate ways to directly increase detection. Concentration of samples after drying and SPE were investigated and it was hypothesised that accuracy would improve by increasing concentration; however findings of the current work refute this hypothesis. Further interpretation shows that concentrating saliva samples over 10 times is not feasible. Firstly, time constraints linked to collection immediately post exercise allowed collection of less than 3 mL saliva ultimately providing concentration restrictions. Secondly, 3 mL SPE cartridges are adequate for up to 1mL saliva and upgrading to 6 mL cartridges would not prove cost effective in this method. Therefore, 1 mL saliva was used for analysis and concentrated 10 times for an end volume of 100 µL. Recovery of analytes may also impact on quantification limits, and SPE produced acceptable recovery after cleaning (>85%). Results revealed that during SPE composition of the wash phase affected cortisol recovery. A methanol wash of 50% significantly decreased recovery of cortisol; however, testosterone recovery was not diminished during this experiment, concurrent with other research (Huq *et al.,* 2008).

Ultimately, the stages of LC-MS method development achieved in Chapters 3 and 4 aimed to produce a valid method to measure salivary cortisol and testosterone levels, and after optimisation, method validation was undertaken. Chapter 5 aimed to validate the method to measure cortisol and testosterone against established validation criteria (ICH, 1996; US Department of Health, 2001). Results showed that the LC-MS method was accurate, precise, linear and produced a LOQ below the lower reference range for cortisol. Inclusion of an internal standard during quantification controlled for variation in injection, sample

preparation, instrument parameters and matrix effects (Magni *et al.*, 2001). Saliva samples taken before and after different exercise trials also showed strong correlation when measured with LC-MS and ELISA (Chapter 6). However, agreement analysis showed poor agreement between the absolute values; this would be expected given that the techniques differ. Some research has suggested that ELISA may lack specificity and overestimate hormone levels therefore this may have contributed to some of the difference.

The significance of this research was developing an LC-MS method to measure cortisol and applying it to measure samples tested in an exercise study. Despite its widespread use in hospitals and the pharmaceutical industry (Kushnir, 2011), LC-MS has not been used to measure salivary cortisol in exercise research. However, testosterone could not be quantified with the current LC-MS method despite extensive optimisation; this was due to lower levels than cortisol in saliva and insufficient sensitivity of the assay. This was established after the LOQ was above the lower reference range for men in saliva and also analysis of salivary samples with LC-MS revealed no correlation with ELISA (Chapter 6). Testosterone exhibits low proton affinity therefore it is harder to ionise than cortisol and low levels can also be difficult to detect. Dervitisation was investigated as a method to improve detection of testosterone; this process changes the chemical structure of a compound and acts to increase proton affinity and detection limits. Hydroxylamine derivatisation of the ketone group of testosterone in our laboratory reported three fold improvements in method sensitivity (changing the m/z from 289 to m/z 304) (Chapter 3), which is slightly lower than the literature reports of 5-10 times (Kushnir *et al*., 2006). Furthermore, LC-MS/MS is also used for detection of small molecules; recent studies have utilised this technique to quantify salivary cortisol and testosterone levels in the reference range for saliva (Matsui *et al.*, 2009; Kusukake *et al.*, 2009). However both studies used the same mass spectrometer (API 4000), with a higher specification than the machine used in the present study, implying the mass spectrometer employed may have contributed to the low detection levels.

During validation, resting salivary samples measured with ELISA showed that unsurprisingly, men had higher testosterone levels than women on average. This is concurrent with other studies (Dabbs, 1990; Bao *et al.*, 2008). However, unexpectedly salivary cortisol was higher in women compared to men. Various reasons for this were proposed included higher stress levels in this group and a more pronounced CAR in

women. A limitation of this study was a lack of questionnaires to establish stress levels which have been shown to affect the CAR (Clow *et al*., 2004). Also, given the low number of participants, expansion of the study would be beneficial to ascertain whether the reported trends are visible within a larger population. Consideration of the CAR was important when planning the exercise validation study as sampling close to awakening may produce a false decrease or negate any exercise induced increase in hormone levels. Sampling in the afternoon and evening has been shown to offer a relatively stable diurnal period for cortisol and testosterone levels (Rose, 1972). Therefore, the exercise study was conducted between 3pm and 8pm.

During the exercise study, salivary cortisol appeared to be linked to RPE. A 30 minute INT session produced a significantly higher RPE and salivary cortisol response than a 30 minute TEMP run and CIR. Furthermore, the TEMP run at lactate threshold (75% VO_{2max}) did not elicit a significant increase in salivary cortisol levels which refutes findings from previous studies (Allgrove et al., 2008; Hill et al., 2008; Budde et al., 2010) reporting a 'threshold intensity' for cortisol release (20 mins, >60% VO_{2max}). However, there is contention over this threshold and differences may relate to the use of percentage VO_{2max} as a controlling variable. Research has postulated that normalising exercise to percentage VO_{2max} may not consider differences in other variables, for example lactate threshold (Coyle *et al*., 1988). Recent research by Lansley *et al*., (2011) investigated an alternative to percentage VO_{2max} for normalisation of exercise intensity, the gas exchange threshold (GET), which is synomomous with lactate threshold. They propose a more accurate normalising variable is %Δ delta GET which is the GET plus percentage interval between the GET and VO_{2max}. This concept could be used to assess the hormonal threshold and remove some discrepancies caused by setting intensity at percentage VO_{2max}. Another finding of the present study was that salivary cortisol levels returned to baseline within 60 minutes of ceasing exercise in all trials except INT. There is evidence to suggest that cortisol enhances lypolysis and protein catabolism to mobilise fuels (Nindl *et al*., (2001), therefore cortisol, may provide a glycogen sparing role during exercise. Studies suggest that the level of glycogen depletion may mediate the hormonal response to exercise rather than the intensity or duration *per se.* There may have been higher glycogen depletion during the interval session, and this could have led to an increased cortisol secretion after this session.

Salivary testosterone showed a post exercise increase after INT, TEMP and CIR sessions. There is evidence that the concomitant increase in cortisol and testosterone secretion after INT may be due to the adrenal glands secreting cortisol and testosterone concurrently (Kroboth *et al.,* 1999). However, salivary testosterone levels rose after CIR without an increase in cortisol concentration. Testosterone may have been released as an anabolic hormone to promote muscle growth and repair after circuit training (Florini, 1985); however, the stress caused by the session may not have been sufficient to elicit enough metabolic demand to require an increase in cortisol and glycogen sparing mechanisms (Nindl *et al*., 2001).

There are no studies examining the hormonal response to aerobic circuit training, although the observed response may reflect reports of increased testosterone levels observed after weight training sessions (Yarrow *et al.,* 2007; Kraemer *et al.,* 1990; Raastad *et al.,* 2000; Hakkinen *et al.,* 1993, Linnamo *et al.,* 2005). The increase in testosterone levels post CIR also suggests that CIR met the proposed threshold of intensity to elicit a testosterone response to resistance training (Vingren *et al.,* 2010). Additional investigation is required to examine VO_{2max} throughout this session to compare CIR with other running training sessions and investigate whether more prolonged training elicits an increase in salivary cortisol levels. Results from Chapter 6 also suggest the salivary C/T ratio response is driven by the cortisol response because an increase in this ratio was only observed after INT.

When examining the peak hormonal response, salivary cortisol levels peaked immediately post TEMP and 15 mins post INT and there was no delay in peak values compared to plasma. These finding refute those suggesting salivary hormone levels peak later than plasma (Kirschbaum and Hellhammer, 1989, 2000; Heinrichs *et al.,* 2001; Hough *et al.,* 2011) and suggests strenuous exercise with multiple HR peaks may contribute to a delayed post exercise peak cortisol response. In studies where a delay in peak salivary cortisol levels were reported, the exercise intensity may have caused an increase in sympathetic activity, leading to temporary vasoconstriction of the arterioles surrounding the salivary glands and decreased blood flow and hormonal diffusion (Chicharro *et al*., 1998). However, the anaerobic threshold postulated to trigger the decrease in salivary flow was likely to be exceeded in both TEMP and INT therefore there may be another reason for the difference in findings. Peak testosterone response occurred immediately post exercise in

saliva and plasma for both INT and TEMP trials. This strengthens the hypothesis that testosterone is secreted through different mechanisms to cortisol; however these mechanisms still remain unclear and require further investigation.

7.2 Conclusions

Based on the findings of this thesis a list of conclusions are summarised as follows:

1. Optimising cone voltage to 25V results in a direct improvement in cortisol (134%) and testosterone (130%) peak area and the reduction of insource fragmentation.

2. Mobile phase flow rate and composition are important to increase ionisation, establish peak resolution and improve analyte signal with optimised flow rate of 0.1 mL/min and a ramped MeOH gradient ranging from 50 to 90% MeOH.

3. Manipulating column temperature between 20 and 50^{o}C does not affect the subsequent peak area of cortisol and testosterone.

4. Sample cleaning methods prior to LC-MS can affect sample recovery, with a reduction in cortisol recovery if the wash phase contains over 50% organic solvent. Analyte polarity is also important during reconstitution of dried sample prior to analysis.

5. Salivary cortisol levels can be measured accurately and precisely with LC-MS, which is supported by validation against established guidelines, including a LOQ of 0.6 ng/mL and correlation with ELISA for saliva samples taken during an exercise intervention study (r=0.93). However, quantification of testosterone in saliva requires further investigation and optimisation.

6. Response of salivary hormones are affected by the intensity and nature of an exercise trial. Increases in salivary cortisol are evident after a 30 minute interval session however they are not significantly changed after a tempo or aerobic circuit session. Testosterone levels are significantly increased after an interval, circuit and tempo session compared to rest.

7. Mean RPE appears to be a good predicator of the salivary cortisol response; illustrated by the significant increase in salivary cortisol levels and higher RPE elicited by the interval session.

8. Plasma and salivary cortisol and testosterone measures were correlated before and after a tempo and interval session supporting the use of salivary measurements to monitor the hormonal response to exercise.

7.3 Future work

Firstly, regarding efficiency of the LC-MS method, development of a UPLC method to reduce analysis time would be more effective and improve sample throughput. UPLC is likely to reduce the run time to <5 minutes. Furthermore, heating the solvent prior to column entry may also reduce analyte retention and sample run time and this could be investigated. Despite the analyte recovery and sample cleaning exhibited by SPE, this method was time consuming and future work could investigate the compatibility of automated SPE to reduce sample preparation time.

The LC-MS method was insufficiently sensitive to quantify testosterone levels in saliva and future research could investigate LC-MS and LC-MS/MS with a saliva matrix to compare differences in S/N ratio with the aim of reducing the LOQ for testosterone. Additionally, derivatisation of testosterone could be investigated further as a method to improve detection during LC-MS.

It would be interesting to investigate the CAR and the mechanisms for the proposed difference between men and women as these are currently unclear. Monitoring VO_{2max} during the exercise trials to measure changes in effort would be useful to give a more in-depth view of the training volume. Finally, there is debate over the use of VO_{2max} to standardise exercise intensity. Therefore, investigation of the acute hormonal response utilising GET and VO_{2max}, may add further insight into the normalisation of exercise intensity and threshold for cortisol and testosterone release.

8. REFERENCES

Aardal, E. and Holm, A. C. (1995). Cortisol in saliva - reference ranges and relation to cortisol in serum. *European Journal of Clinical Chemistry and Clinical Biochemistry* **33**: 927-932.

Abaye, D. A., Pullen, F. S. and Nielsen, B. V. (2011). Peptide polarity and the position of arginine as sources of selectivity during positive electrospray ionisation mass spectrometry. *Rapid Communications in Mass Spectrometry* **25**: 3597-3608.

Abaye, D. A., Pullen, F. S. and Nielsen, B. V. (2011). Practical considerations in analysing neuropeptides, calcitonin gene-related peptide and vasoactive intestinal peptide, by nano-electrospray ionisation and quadrupole time-of-flight mass spectrometry: Monitoring multiple protonations. *Rapid Communications in Mass Spectrometry* **25**: 1107-1116.

ABSciex. (2010). API 4000™ LC-MS/MS system. Retrieved September, 2011, from http://www.absciex.com/Documents/Downloads/Literature/mass-spectrometry-cms_039796.pdf.

AbuRuz, S., Millership, J., Heaney, L. and McElnay, J. (2003). Simple liquid chromatography method for the rapid simultaneous determination of prednisolone and cortisol in plasma and urine using hydrophilic lipophilic balanced solid phase extraction cartridges. *Journal of Chromatography B* **798**: 193-201.

Adlercreutz, H., Harkonen, M., Kuoppasalmi, K., Naveri, H., Huhtaniemi, I., Tikkanen, H., Remes, K., Dessypris, A. and Karvonen, J. (1986). Effect of training on plasma anabolic and catabolic steriod hormones and their response to physical exercise. *International Journal of Sports Medicine* **7**: 27-28.

Ahtiainen, J. P., Pakarinen, A., Alen, M., Kraemer, W. J. and Hakkinen, K. (2005). Short vs. Long rest period between the sets in hypertrophic resistance training: Influence on muscle strength, size, and hormonal adaptations in trained men. *Journal of Strength and Conditioning Research* **19**: 572-82.

Ahtiainen, J. P., Pakarinen, A., Kraemer, W. J. and Hakkinen, K. (2004). Acute hormonal responses to heavy resistance exercise in strength athletes versus nonathletes. *Canadian Journal of Applied Physiology* **29**: 527-43.

Alexander, M. S. and Dolan, J. W. (2009). *LC-MS/MS for chromatographers*, LC Resources Inc.

Allgrove, J. E., Gomes, E., Hough, J. and Gleeson, M. (2008). Effects of exercise intensity on salivary antimicrobial proteins and markers of stress in active men. *Journal of Sports Sciences* **26**: 653 - 661.

Annesley, T. M. (2003). Ion suppression in mass spectrometry. *Clinical Chemistry* **49**: 1041-1044.

Argus, C. K., Gill, N. D., Keogh, J. W., Hopkins, W. G. and Beaven, C. M. (2009). Changes in strength, power, and steroid hormones during a professional rugby union competition. *Journal of Strength and Conditioning Research* **23**: 1583-92.

Avery, M. J. (2003). Quantitative characterization of differential ion suppression on liquid chromatography/atmospheric pressure ionisation mass spectrometric bioanalytical methods. *Rapid Communications in Mass Spectrometry* **17**: 197-201.

Axelrod, J. and Reisine, T. D. (1984). Stress hormones: Their interaction and regulation. *Science* **224**: 452-9.

Baid, S. K., Sinaii, N., Wade, M., Rubino, D. and Nieman, L. K. (2007). Radioimmunoassay and tandem mass spectrometry measurement of bedtime salivary cortisol levels: A comparison of assays to establish hypercortisolism. *Journal of Clinical Endocrinology and Metabolism* **92**: 3102-3107.

Bambino, T. H. and Hsueh, A. J. (1981). Direct inhibitory effect of glucocorticoids upon testicular luteinizing hormone receptor and steroidogenesis in vivo and in vitro. *Endocrinology* **108**: 2142-8.

Banfi, G., Marinelli, M., Roi, G. S. and Agape, V. (1993). Usefulness of free testosterone/cortisol ratio during a season of elite speed skating athletes. *International Journal of Sports Medicine* **14**: 373-9.

Bao, S., Peng, Y., Sheng, S. and Lin, Q. (2008). Assessment of urinary total testosterone production by a highly sensitive time-resolved fluorescence immunoassay. *Journal of Clinical Laboratory Analysis* **22**: 403-8.

Bardon, A., Ceder, O. and Kollberg, H. (1983). Cystic fibrosis-like changes in saliva of healthy persons subjected to anaerobic exercise. *Clinica Chimica Acta* **133**: 311-316.

Baxendale, P. M., Reed, M. J. and James, V. H. T. (1980). Testosterone in saliva of normal men and its relationship with unbound and total testosterone levels in plasma. *The Journal of Endocrinology* **87**: 46.

Beaven, M. C., Gill, N. D. and Cook, C. J. (2008). Salivary testosterone and cortisol responses in professional rugby players after four resistance exercise protocols. *Journal of Strength and Conditioning Research* **22**: 426-432.

Benijts, T., Dams, R., Lambert, W. and De Leenheer, A. (2004). Countering matrix effects in environmental liquid chromatography electrospray ionisation tandem mass spectrometry water analysis for endocrine disrupting chemicals. *Journal of Chromatography A* **1029**: 153-159.

Bertram, U. (1967). Xerostomia. Clinical aspects, pathology and pathogenesis. *Acta Odontol Scand.* **25**: 1-126.

Bland, J. M. and Altman, D. G. (1986). Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* **8**: 307-310.

Bonfiglio, R., King, R. C., Olah, T. V. and Merkle, K. (1999). The effects of sample preparation methods on the variability of the electrospray ionisation response for model drug compounds. *Rapid Communications in Mass Spectrometry* **13**: 1175-1185.

Booth, A., Johnson, D. R., Granger, D. A., Crouter, A. C. and McHale, S. (2003). Testosterone and child and adolescent adjustment: the moderating role of parent-child relationships. *Developmental Psychology* **39**: 85-98.

Borrey, D. l., Moerman, E., Cockx, A., Engelrelst, V. and Langlois, M. R. (2007). Column-switching LC-MS/MS analysis for quantitative determination of testosterone in human serum. *Clinica Chimica Acta* **382**: 134-137

Bowden, J. A., Colosi, D. M., Mora-Montero, D. C., Garrett, T. J. and Yost, R. A. (2009). Enhancement of chemical derivatization of steroids by gas chromatography/mass spectrometry (GC/MS*) Journal of Chromatography B* **877**: 3237-3242.

Bowermaster, J. and McNair, H. M. (1984). Temperature programmed microbore HPLC part i. *Journal of Chromatographic Science* **22**: 165-170.

Boyd, R. K., Basic, C. and Bethem, R. A. (2008). *Trace quantitative analysis by mass spectrometry*. Chichester, Wiley.

Brambilla, D. J., Matsumoto, A. M., Araujo, A. B. and McKinlay, J. B. (2009). The effect of diurnal variation on clinical measurement of serum testosterone and other sex hormone levels in men. *Journal of Clinical Endocrinology & Metabolism* **94**: 907-13.

Braunwald, E., Fauci, A. S., Kasper, D. L., Hauser, S. L., Longo, D. L., Jameson, J. L. and Loscalzo, J. (2001). *Harrison's principles of internal medicine* York, McGraw-Hill.

Bremner, W. J., Vitiello, M. V. and Prinz, P. N. (1983). Loss of circadian rhythmicity in blood testosterone levels with aging in normal men. *Journal of Clinical Endocrinology & Metabolism* **56**: 1278-1281.

Bricout, V. A., Serrurier, B. D., Bigard, A. X. and Guezennec, C. Y. (1999). Effects of hindlimb suspension and androgen treatment on testosterone receptors in rat skeletal muscles. *European Journal of Applied Physiology and Occupational Physiology* **79**: 443-8.

Brook, C. G. D. and Marshall, N. J. (2001). *Essential endocrinology*. Oxford, Blackwell.

Brownlee, K. K., A.W, M. and Hackney, A. C. (2005). Relationship between circulating cortisol and testosterone: influence of physical exercise. *Journal of Sports Science and Medicine* **4**: 76-83.

Brownlee, K. K., Viru, M., Viru, A., Behr, M. and Hackney, A. C. (2006). Exercise and the relationship between circulating cortisol and testosterone concentrations in men. *Physical Education and Sport* **50**: 30-33.

Bruins, A. P. (1991). Mass spectrometry with ion sources operating at atmospheric pressure. *Mass Spectrometry Reviews* **10**: 53-77.

Budde, H., Voelcker-Rehage, C., Pietrassyk-Kendziorra, S., Machado, S., Ribeiro, P. and Arafat, A. M. (2010). Steroid hormones in the saliva of adolescents after different exercise intensities and their influence on working memory in a school setting. *Psychoneuroendocrinology* **35**: 382-391.

Budgett, R. (1990). Overtraining syndrome. *British Journal of Sports Medicine* **24**: 231-236.

Budgett, R. (1998). Fatigue and underperformance in athletes: The overtraining syndrome. *British Journal of Sports Medicine* **32**: 107-10.

Buhrman, D. L., Price, P. I. and Rudewicz, P. J. (1996). Quantitation of sr 27417 in human plasma using electrospray liquid chromatography-tandem mass spectrometry: A study of ion suppression. *Journal of the American Society for Mass Spectrometry* **7**: 1099-1105.

Busch, K. L., Glish, G. L. and McLuckey, S. A. (1988). *Mass spectrometry: Techniques and applications of tandem mass spectrometry* VCH: New York.

Cadore, E., Lhullier, F., Brentano, M., Silva, E., Ambrosini, M., Spinelli, R., Silva, R. and Kruel, L. (2008). Correlations between serum and salivary hormonal concentrations in response to resistance exercise. *Journal of Sports Sciences* **26**: 1067 - 1072.

Cadore, E. L., Lhullier, F. L., Alberton, C. L., Almeida, A. P., Sapata, K. B., Korzenowski, A. L. and Kruel, L. F. (2009). Salivary hormonal responses to different water-based exercise protocols in young and elderly men. *Journal of Strength and Conditioning Research* **23**: 2695-701.

Cadore, E. L., Lhullier, F. L., Brentano, M. A., da Silva, E. M., Ambrosini, M. B., Spinelli, R., Silva, R. F. and Kruel, L. F. (2008). Hormonal responses to resistance exercise in long-term trained and untrained middle-aged men. *Journal of Strength and Conditioning Research* **22**: 1617-24.

Cannon, W. (1935). Stress and strains of homeostasis. *American Journal of Medicine* **189**: 1-14.

Carroll, D. (1992). *Health psychology: Stress behaviour and disease*. London, Falmer.

Castro, W. L. and Matt, K. S. (1997). Neuroendocrine correlates of separation stress in the siberian dwarf hamster (phodopus sungorus). *Physiology and Behaviour* **61**: 477-84.

Cawood, M. L., Field, H. P., Ford, C. G., Gillingwater, S., Kicman, A., Cowan, D. and Barth, J. H. (2005). Testosterone measurement by isotope-dilution liquid chromatography-tandem mass spectrometry: Validation of a method for routine clinical practice. *Clinical Chemistry* **51**: 1472-1479.

Chang, Y., Li, C., Li, A., Jong, S., Liao, P. and Chang, L. W. (2003). Quantitative measurement of male steroid hormones using automated on-line solid phase extraction-liquid chromatography-tandem mass spectrometry and comparison with radioimmunoassay. *The Analyst* **128**: 363-368.

Chen, Y., Yazdanpanah, M., Hoffman, B. R., Diamandis, E. P. and Wong, P.-Y. (2009). Rapid determination of serum testosterone by liquid chromatography-isotope dilution tandem mass spectrometry and a split sample comparison with three automated immunoassays. *Clinical Biochemistry* **42**: 484-490.

Chicharro, J. L., Lucia, A., Perez, M., Vaquero, A. F. and Urena, R. (1998). Saliva composition and exercise. *Sports Medicine* **26**: 17-27.

Chrousos, G. P. (1998). Stressors, stress, and neuroendocrine integration of the adaptive response: The 1997 hans selye memorial lecture. *Annals of the New York Academy of Sciences* **851**: 311-335.

Clow, A., Thorn, L., Evans, P. and Hucklebridge, F. (2004). The awakening cortisol response: Methodological issues and significance. *Stress* **7**: 29-37.

Cole, R. B. (1997). *Electrospray ionisation mass spectrometry : Fundamentals, instrumentation, and applications* New York, Wiley

Cole, R. B. (2000). Some tenets pertaining to electrospray ionisation mass spectrometry. *Journal of Mass Spectrometry* **35**: 763-772.

Comoglio, S. and Celada, F. (1976). An immuno-enzymatic assay of cortisol using e. Coli î²-galactosidase as label. *Journal of Immunological Methods* **10**: 161-170.

Cordova, A., Sureda, A., Tur, J. A. and Pons, A. (2010). Immune response to exercise in elite sportsmen during the competitive season. *Journal of Physiology and Biochemistry* **66**: 1-6.

Costill, D. L., Gollnick, P. D., Jansson, E. D., Saltin, B. and Stein, E. M. (1973). Glycogen depletion pattern in human muscle fibres during distance running. *Acta Physiologica Scandinavica* **89**: 374-383.

Coutts, A. J., Reaburn, P., Piva, T. J. and Rowsell, G. J. (2007). Monitoring for overreaching in rugby league players. *European Journal of Applied Physiology* **99**: 313-24.

Coyle, E. F., Coggan, A. R., Hemmert, M. K. and Ivy, J. L. (1986). Muscle glycogen utilisation during prolonged strenuous exercise when fed carbohydrate. *Journal of Applied Physiology* **61**: 165-172.

Coyle, E. F., Coggan, A. R., Hopper, M. K. and Walters, T. J. (1988). Determinants of endurance in well-trained cyclists. *Journal of Applied Physiology* **64**: 2622-2630.

Crewther, B., Keogh, J., Cronin, J. and Cook, C. (2006). Possible stimuli for strength and power adaptation: Acute hormonal responses. *Sports Medicine* **36**: 215-38.

Cumming, D. C., Quigley, M. E. and Yen, S. S. C. (1983). Acute suppression of circulating testosterone levels by cortisol in men. *Journal of Clinical Endocrinology & Metabolism* **57**: 671-673.

Dabbs, J. M. (1990). Salivary testosterone measurements: reliability across hours, days and weeks. *Physiology and Behaviour* **48**: 83-86.

Dabbs, J. M. J. (1991). Salivary testosterone measurements: Collecting, storing, and mailing saliva samples. *Physiology and Behaviour* **49**: 815-817.

Daly, W., Seegers, C. A., Rubin, D. A., Dobridge, J. D. and Hackney, A. C. (2005). Relationship between stress hormones and testosterone with prolonged endurance exercise. *European Journal of Applied Physiology* **93**: 375-380.

Dawes, C. (1981). The effects of exercise on protein and electrolyte secretion in parotid saliva. *The Journal of Physiology* **320**: 139-148.

De Palo, E. F., Antonelli, G., Benetazzo, A., Prearo, M. and Gatti, R. (2009). Human saliva cortisone and cortisol simultaneous analysis using reverse phase hplc technique. *Clinica Chimica Acta* **405**: 60-65.

del Corral, P., Mahon, A. D., Duncan, G. E., Howe, C. A. and Craig, B. W. (1994). The effect of exercise on serum and salivary cortisol in male children. *Medicine & Science in Sports & Exercise* **26**: 1297-301.

Dempster, A. J. (1921). Positive ray analysis of lithium and magnesium. *Physical Review* **18**: 415-422.

Di Luigi, L., Guidetti, L., Baldari, C., Gallotta, M. C., Sgro, P., Perroni, F., Romanelli, F. and Lenzi, A. (2006). Cortisol, dehydroepiandrosterone sulphate and dehydroepiandrosterone sulphate/cortisol ratio responses to physical stress in males are influenced by pubertal development. *Journal of Endocrinological Investigation* **29**: 796-804.

DiFrancesco, R., Frerichs, V., Donnelly, J., Hagler, C., Hochreiter, J. and Tornatore, K. M. (2007). Simultaneous determination of cortisol, dexamethasone, methylprednisolone, prednisone, prednisolone, mycophenolic acid and mycophenolic acid glucuronide in human plasma utilizing liquid chromatography tandem mass spectrometry. *Journal of Chromatography B* **859**: 42-51.

Dimitriou, L., Sharp, N. C. and Doherty, M. (2002). Circadian effects on the acute responses of salivary cortisol and IgA in well trained swimmers. *British Journal of Sports Medicine* **36**: 260-4.

Diver, M. J., Imtiaz, K. E., Ahmad, A. M., Vora, J. P. and Fraser, W. D. (2003). Diurnal rhythms of serum total, free and bioavailable testosterone and of SHBG in middle-aged men compared with those in young men. *Clinical Endocrinology* **58**: 710-7.

Dolan, J. W. (2002). Temperature selectivity in reversed-phase high performance liquid chromatography. *Journal of Chromatography A* **965**: 195-205.

Dolan, J. W. (2010). Column diameter, linear velocity and column efficiency. *LCGC Asia Pacific* **13**: Epub Mar 1, 2010.

Dole, M., Mack, L. L., Hines, R. L., Mobley, R. C., Ferguson, L. D. and Alice, M. B. (1968). Molecular beams of macroions. *Journal of Chemical Physics* **49**: 2240-2249.

Dufau, M. L., Catt, K. J., Tsuruhara, T. and Ryan, D. (1972). Radioimmunoassay of plasma testosterone. *Clinica Chimica Acta* **37**: 109-116.

Dvorak, J., Feddermann, N. and Grimm, K. (2006). Glucocorticosteroids in football: use and misuse. *British Journal of Sports Medicine* **40**: i48-i54.

Dyson, N. A. (1998). *Chromatographic integration methods*, Royal Society of Chemistry: Cambridge.

Eik-Nes, K. B. (1969). An effect of isoproterenol on rates of synthesis and secretion of testosterone. *The American journal of physiology* **217**: 1764-70.

Eliakim, A., Portal, S., Zadik, Z., Rabinowitz, J., Adler-Portal, D., Cooper, D. M., Zaldivar, F. and Nemet, D. (2009). The effect of a volleyball practice on anabolic hormones and inflammatory markers in elite male and female adolescent players. *Journal of Strength and Conditioning Research* **23**: 1553-9.

Elloumi, M., Maso, F., Michaux, O., Robert, A. and Lac, G. (2003). Behaviour of saliva cortisol [c], testosterone [t] and t/c ratio during a rugby match and during the post-competition recovery days. *European Journal of Applied Physiology* **90**: 23-8.

Enea, C., Boisseau, N., Ottavy, M., Mulliez, J., Millet, C., Ingrand, I., Diaz, V. and Dugue, B. (2009). Effects of menstrual cycle, oral contraception, and training on exercise-induced changes in circulating dhea-sulphate and testosterone in young women. *European Journal of Applied Physiology* **106**: 365-73.

Engvall, E. and Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* **8**: 1-4.

Enoki, T., Yoshida, Y., Lally, J., Hatta, H. and Bonen, A. (2006). Testosterone increases lactate transport, monocarboxylate transporter (MCT) 1 and MCT4 in rat skeletal muscle. *Journal of Physiology* **577**: 433-43.

Fahrner, C. L. and Hackney, A. C. (1998). Effects of endurance exercise on free testosterone concentration and the binding affinity of sex hormone binding globulin (SHBG). *International Journal of Sports Medicine* **19**: 12,15.

Faiman, C. and Winter, J. S. D. (1971). Diurnal cycles in plasma FSH, testosterone and cortisol in men. *The Journal of Clinical Endocrinology & Metabolism* **33**: 186-192.

Fanelli, F., Belluomo, I., Di Lallo, V. D., Cuomo, G., De Iasio, R., Baccini, M., Casadio, E., Casetta, B., Vicennati, V., Gambineri, A., Grossi, G., Pasquali, R. and Pagotto, U. (2010). Serum steroid profiling by isotopic dilution-liquid chromatography mass spectrometry: Comparison with current immunoassays and reference intervals in healthy adults. *Steroids* **76**: 244-253.

Fang, K., Pan, X.-J., Huang, B., Liu, J.-L., Wang, Y. and Gao, J.-P. (2010). Progress on keto groups derivatization of steroid hormones in gas chromatography-mass spectrometry analysis. *Chinese Journal of Analytical Chemistry* **38**: 743-751.

Farzanaki, P., Azarbayjani, M. A., Rasaee, M. J., Jourkesh, M., Ostojic, S. M. and Stannard, S. (2008). Salivary immunoglobulin A and cortisol response to training in young elite female gymnasts. *Brazilian Journal of Biomotricity* **2**: 254-258.

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. and Whitehouse, C. M. (1990). Electrospray ionisation–principles and practice. *Mass Spectrometry Reviews* **9**: 37-70.

Fitzgerald, R. and Herold, D. (1996). Serum total testosterone: immunoassay compared with negative chemical ionisation gas chromatography-mass spectrometry. *Clinical Chemistry* **42**: 749-755.

Fitzgerald, R. L., Griffin, T. L. and Herold, D. A. (2010). Analysis of testosterone in serum using mass spectrometry. *Methods in Molecular Biology* **603**: 489-500.

Florini, J. R. (1985). Hormonal control of muscle cell growth. *Journal of Animal Science* **61**:21-38.

Ford, H., Johnson, L., Purdie, G. and Feek, C. (1997). Effects of hyperthyroidism and radioactive iodine given to ablate the thyroid on the composition of whole stimulated saliva. *Clinical Endocrinology* **46**: 189-193.

Franca, S. C., Barros Neto, T. L., Agresta, M. C., Lotufo, R. F. and Kater, C. E. (2006). [divergent responses of serum testosterone and cortisol in athlete men after a marathon race]. *Arquivos Brasileiros de Endocrinologia & Metabologia* **50**: 1082-7.

Fry, A. and Lohnes, C. (2010). Acute testosterone and cortisol responses to high power resistance exercise. *Human Physiology* **36**: 457-461.

Furuyama, S., Mayes, D. and Nugent, C. (1970). A radioimmunoassay for plasma testosterone. *Steroids* **16**: 415-428.

Galbo, H. (2001). Influence of aging and exercise on endocrine function. *International Journal of Sports Nutrition and Exercise Metabolism* **11**: S49-57.

Galbo, H., Hummer, L., Peterson, I. B., Christensen, N. J. and Bie, N. (1977). Thyroid and testicular hormone responses to graded and prolonged exercise in man. *European Journal of Applied Physiology and Occupational Physiology* **36**: 101-6.

Gale, D. C. and Smith, R. D. (1993). Small volume and low flow-rate electrospray ionisation mass spectrometry of aqueous samples. *Rapid Communications in Mass Spectrometry* **7**: 1017-1021.

Gallagher, L. M., Owen, L. J. and Keevil, B. G. (2007). Simultaneous determination of androstenedione and testosterone in human serum by liquid chromatography-tandem mass spectrometry. *Annals of Clinical Biochemistry* **44**: 48-56.

Garces, C., de Oya, I., Lopez-Simon, L., Cano, B., Schoppen, S., Gil, A. and de Oya, M. (2008). Hormone levels in 12- to 15-year-old boys and girls in Spain and their relationship with anthropometric variables. *Clinical Biochemistry* **41**: 621-4.

Gohlke, R. S. (1959). Time-of-flight mass spectrometry and gas-liquid partition chromatography. *Analytical Chemistry* **31**: 535-541.

Gohlke, R. S. (1959). Time-of-flight mass spectrometry and gas-liquid partition chromatography. *Analytical Chemistry* **31**: 535-541.

González-Bono, E., Moya-Albiol, L., Martínez-Sanchis, S. and Salvador, A. (2002). Salivary testosterone and cortisol responses to cycle ergometry in basketball players with different training volume. *Journal of Psychophysiology* **16**: 158-166.

Granger, D. A., Shirtcliff, E. A., Booth, A., Kivlighan, K. T. and Schwartz, E. B. (2004). The trouble with salivary testosterone. *Psychoneuroendocrinology* **29**: 1229-1240.

Grant, D. A., Stem, I. B. and Listgarten, M. A. (1988). Saliva. *Peridontics*. St Louis, CV Mosby**:** 135-146.

Grego, F., Vallier, J.-M., Collardeau, M., Bermon, S., Ferrari, P., Candito, M., Bayer, P., Magnié, M.-N. and Brisswalter, J. (2004). Effects of long duration exercise on cognitive function, blood glucose, and counterregulatory hormones in male cyclists. *Neuroscience Letters* **364**: 76-80.

Gröschl, M. and Rauh, M. (2006). Influence of commercial collection devices for saliva on the reliability of salivary steroids analysis. *Steroids* **71**: 1097-1100.

Gulledge, T. and Hackney, A. (1996). Reproducibility of low resting testosterone concentrations in endurance trained men. *European Journal of Applied Physiology and Occupational Physiology* **73**: 582-583.

Guo, T., Taylor, R. L., Singh, R. J. and Soldin, S. J. (2006). Simultaneous determination of 12 steroids by isotope dilution liquid chromatography-photospray ionisation tandem mass spectrometry. *Clinica Chimica Acta* **372**: 76-82.

Hackney, A. C. and Dobridge, J. (2003). Exercise and male hypogonadism: Testosterone, the hypothalamic-pituitary-testicular axis, and physical exercise *Male hypogonadism: Basic, clinical, and therapeutic principles*. S. Winters. Totowa, N.J, Humana Press**:** 305–330.

Hackney, A. C. and Viru, A. (1999). Twenty-four-hour cortisol response to multiple daily exercise sessions of moderate and high intensity. *Clinical Physiology* **19**: 178-82.

Hakkinen, K. and Pakarinen, A. (1993). Acute hormonal responses to two different fatiguing heavy-resistance protocols in male athletes. *Journal of Applied Physiology* **74**: 882-7.

Hakkinen, K., Pakarinen, A., Alen, M., Kauhanen, H. and Komi, P. V. (1987). Relationships between training volume, physical performance capacity, and serum hormone concentrations during prolonged training in elite weight lifters. *International Journal of Sports Medicine* **1**: 61-5.

Hansen, M., Jacobsen, N. W., Nielsen, F. K., Bjorklund, E., Styrishave, B. and Halling-Sorensen, B. (2011). Determination of steroid hormones in blood by GC-MS/MS. *Analytical and Bioanalytical Chemistry* **400**: 3409-17.

Harman, S. M., Metter, E. J., Tobin, J. D., Pearson, J. and Blackman, M. R. (2001). Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore longitudinal study of aging. *Journal of Clinical Endocrinology & Metabolism* **86**: 724-31.

Harris, A., Marquis, P., Eriksen, H. R., Grant, I., Corbett, R., Lie, S. A. and Ursin, H. (2010). Diurnal rhythm in british antarctic personnel. *Rural Remote Health* **10**: 18.

Havlikova, L., Matysova, L., Hajkova, R., Satinsky, D. and Solich, P. (2008). Advantages of pentafluorophenylpropyl stationary phase over conventional C18 stationary phase--application to analysis of triamcinolone acetonide. *Talanta* **76**: 597-601.

Hawker, C. D. (1973). Radioimmunoassay and related methods. *Analytical Chemistry* **45**: 878A-890a.

Heinrichs, M., Meinlschmidt, G., Neumann, I., Wagner, S., Kirschbaum, C., Ehlert, U. and Hellhammer, D. H. (2001). Effects of suckling on hypothalamic-pituitary-adrenal axis responses to psychosocial stress in postpartum lactating women. *Journal of Clinical Endocrinology & Metabolism* **86**: 4798-4804.

Heintze, U., Birkhed, D. and Bjorn, H. (1983). Secretion rate and buffer effect of resting and stimulated whole saliva as a function of age and sex. *Swedish Dental Journal* **7**: 227-38.

Hellhammer, D. H., Wüst, S. and Kudielka, B. M. (2009). Salivary cortisol as a biomarker in stress research. *Psychoneuroendocrinology* **34**: 163-171.

Hellman, L., Nakada, F., Curti, J., Weitzman, E. D., Kream, J., Roffwarg, H., Ellman, S., Fukushima, D. K. and Gallagher, T. F. (1970). Cortisol is secreted episodically by normal man. *The Journal of Clinical Endocrinology & Metabolism* **30**: 411-422.

Herold, D. A. and Fitzgerald, R. L. (2003). Immunoassays for testosterone in women: Better than a guess? *Clinical Chemistry* **49**: 1250-1251.

Higashi, T. and Shimada, K. (2004). Derivatisation of neutral steroids to enhance their detection characteristics in liquid chromatography mass spectrometry. *Analytical and Bioanalytical Chemistry* **378**: 875-882.

Higashi, T., Yamauchi, A. and Shimada, K. (2005). 2-hydrazino-1-methylpyridine: A highly sensitive derivatization reagent for oxosteroids in liquid chromatography-electrospray ionisation-mass spectrometry. *Journal of Chromatography B* **825**: 214-222.

Hill, E. E., Zack, E., Battaglini, C., Viru, M., Viru, A. and Hackney, A. C. (2008). Exercise and circulating cortisol levels: the intensity threshold effect. *Journal of Endocrinological Investigation* **31**: 587-91.

Hogg, C. J., Vickers, E. R. and Rogers, T. L. (2005). Determination of testosterone in saliva and blow of bottlenose dolphins (*tursiops truncatus*) using liquid chromatography-mass spectrometry. *Journal of Chromatography B* **814**: 339-346.

Holloszy, J. O. and Coyle, E. F. (1984). Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. *Journal of Applied Physiology* **56**: 831-838.

Hong, H. S. (2008). Measurement of testosterone level in LOH Retrieved April, 2011, from http://urologycentre.com.sg/hypogonadism_testosteronelevel.html.

Horowitz, J. F. and Klein, S. (2000). Lipid metabolism during endurance exercise. *The American Journal of Clinical Nutrition* **72**: 558S-563S.

Hough, J. P., Papacosta, E., Wraith, E. and Gleeson, M. (2011). Plasma and salivary steroid hormone responses of men to high-intensity cycling and resistance exercise. *Journal of Strength and Conditioning Research* **25**: 23-31.

Humphrey, S. P. and Williamson, R. T. (2001). A review of saliva: normal composition, flow, and function. *The Journal of Prosthetic Dentistry* **85**: 162-169.

Huq, S., Kallury, K. and Campognone, M. (2008). Optimisation of wash/elution conditions for automated solid-phase extraction (SPE) of testosterone from plasma. *Phenomenex Applications* **TN-007**

ICH (1996). *Validation of analytical procedures: Text and methodology*. International Conference on Harminisaton of Technical Requirements for Registration of Pharmaceuticals for Human Use.

Ionita, I. A. and Akhlaghi, F. (2010). Quantification of unbound prednisolone, prednisone, cortisol and cortisone in human plasma by ultrafiltration and direct injection into liquid chromatrography tandem mass spectrometry. *Annals of Clinical Biochemistry* **47**: 350-357.

Ionita, I. A., Fast, D. M. and Akhlaghi, F. (2009). Development of a sensitive and selective method for the quantitative analysis of cortisol, cortisone, prednisolone and prednisone in human plasma. *Journal of Chromatography B* **877**: 765-772.

Iribarne, J. V. and Thomson, B. A. (1976). On the evaporation of small ions from charged droplets. *Journal of Chemical Physics* **64**: 2287-2294.

Ishigaki, T., Koyama, K., Tsujita, J., Tanaka, N., Hori, S. and Oku, Y. (2005). Plasma leptin levels of elite endurance runners after heavy endurance training. *Journal of Physiological Anthropology and Applied Human Science* **24**: 573-8.

Isotopes, I. o. (2011). *Cortisol RIA kit insert*.

Ispirlidis, I., Fatouros, I. G., Jamurtas, A. Z., Nikolaidis, M. G., Michailidis, I., Douroudos, I., Margonis, K., Chatzinikolaou, A., Kalistratos, E., Katrabasas, I., Alexiou, V. and Taxildaris, K. (2008). Time-course of changes in inflammatory and performance responses following a soccer game. *Clinical Journal of Sports Medicine* **18**: 423-31.

Jacks, D. E., Sowash, J., Anning, J., McGloughlin, T. and Andres, F. (2002). Effect of exercise at three exercise intensities on salivary cortisol. *Journal of Strength and Conditioning Research* **16**: 286-289.

Jessop, D. S. and Turner-Cobb, J. M. (2008). Measurement and meaning of salivary cortisol: a focus on health and disease in children. *Stress: The International Journal on the Biology of Stress* **11**: 1-14.

Jezova, D. and Vigas, M. (1981). Testosterone response to exercise during blockade and stimulation of adrenergic receptors in man. *Hormone Research* **15**: 141-7.

Jiao, J., Carella, A. J., Steeno, G. S. and Darrington, R. T. (2002). Optimisation of triple quadrupole mass spectrometer for quantitation of trace degradants of pharmaceutical compounds. *International Journal of Mass Spectrometry* **216**: 209-218.

Jones, A. M. and Carter, H. (2000). The effect of endurance training on parameters of aerobic fitness. *Sports Medicine* **29**: 373-386.

Jones, A., Marin, S., Clark, J. and Porter, N. (2005). Proceedings 29th international symposium on high performance liquid phase separations and related techniques. *Journal of Chromatography A* **1119**: 1-321.

Jönsson, B. A. G., Malmberg, B., Amilon, Å., Helene Garde, A. and Ørbæk, P. (2003). Determination of cortisol in human saliva using liquid chromatography-electrospray tandem mass spectrometry. *Journal of Chromatography B* **784**: 63-68.

Juhan, D. (1998). *A handbook for bodywork: Job's body*. NY, Barrytown Ltd.

Kalhorn, T. F., Page, S. T., Howald, W. N., Mostaghel, E. A. and Nelson, P. S. (2007). Analysis of testosterone and dihydrotestosterone from biological fluids as the oxime derivatives using high-performance liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **21**: 3200-3206.

Karkoulias, K., Habeos, I., Charokopos, N., Tsiamita, M., Mazarakis, A., Pouli, A. and Spiropoulos, K. (2008). Hormonal responses to marathon running in non-elite athletes. *European Journal of Internal Medicine* **19**: 598-601.

Kataoka, H., Matsuura, E. and Mitani, K. (2007). Determination of cortisol in human saliva by automated in-tube solid-phase microextraction coupled with liquid chromatography-mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **44**: 160-165.

Khosla, S., Melton, L. J., 3rd, Atkinson, E. J. and O'Fallon, W. M. (2001). Relationship of serum sex steroid levels to longitudinal changes in bone density in young versus elderly men. *Journal of Clinical Endocrinology and Metabolism* **86**: 3555-61.

King, R., Bonfiglio, R., Fernandez-Metzler, C., Miller-Stein, C. and Olah, T. (2000). Mechanistic investigation of ionisation suppression in electrospray ionisation. *Journal of the Amercian Society of Mass Spectrometry* **11**: 942-50.

Kirschbaum, C. and Hellhammer, D. H. (1989). Salivary cortisol in psychobiological research: An overview. *Neuropsychbiology* **22**: 150-169.

Kirschbaum, C. and Hellhammer, D. H. (1994). Salivary cortisol in psychoendocrine research: Recent developements and applications. *Psychoneuroendocrinology* **19**: 313-333.

Kirschbaum, C. and Hellhammer, D. H. (2000). Salivary cortisol. *Encyclopedia of stress*. G. Fink. San Diego, Academic Press. **3:** 379-383.

Kirschbaum, C., Kudielka, B. M., Gaab, J., Schommer, N. C. and Hellhammer, D. H. (1999). Impact of gender, menstrual cycle phase, and oral contraceptives on the activity of the hypothalamus-pituitary-adrenal axis. *Psychosomatic medicine* **61**: 154-162.

Kivlighan, K. T., Granger, D. A., Schwartz, E. B., Nelson, V., Curran, M. and Shirtcliff, E. A. (2004). Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Hormones and Behaviour* **46**: 39-46.

Kokalas, N., Tsalis, G., Tsigilis, N. and Mougios, V. (2004). Hormonal responses to three training protocols in rowing. *European Journal of Applied Physiology* **92**: 128-32.

Korenman, S. G., Morley, J. E., Mooradian, A. D., Davis, S. S., Kaiser, F. E., Silver, A. J., Viosca, S. P. and Garza, D. (1990). Secondary hypogonadism in older men: Its relation to impotence. *Journal of Clinical Endocrinology and Metabolism* **71**: 963-9.

Kraemer, W. J., Dziados, J. E., Marchitelli, L. J., Gordon, S. E., Harman, E. A., Mello, R., Fleck, S. J., Frykman, P. N. and Triplett, N. T. (1993). Effects of different heavy-resistance exercise protocols on plasma beta-endorphin concentrations. *Journal of Applied Physiology* **74**: 450-9.

Kraemer, W. J., Marchitelli, L., Gordon, S. E., Harman, E., Dziados, J. E., Mello, R., Frykman, P., Mccurry, D. and Fleck, S. J. (1990). Hormonal and growth factor responses to heavy resistance exercise protocols. *Journal of Applied Physiology* **69**: 1442-1450.

Kraemer, W. J., Fleck, S. J., Maresh, C. M., Ratamess, N. A., Gordon, S. E., Goetz, K. L., Harman, E. A., Frykman, P. N., Volek, J. S., Mazzetti, S. A., Fry, A. C., Marchitelli, L. J. and Patton, J. F. (1999). Acute hormonal responses to a single bout of heavy resistance exercise in trained power lifters and untrained men. *Canadian Journal of Applied Physiology* **24**: 524-37.

Kraemer, W. J., Gordon, S. E., Fleck, S. J., Marchitelli, L. J., Mello, R., Dziados, J. E., Friedl, K., Harman, E., Maresh, C. and Fry, A. C. (1991). Endogenous anabolic hormonal and growth factor responses to heavy resistance exercise in males and females. *International Journal of Sports Medicine* **12**: 228-35.

Kraemer, W. J., Loebel, C. C., Volek, J. S., Ratamess, N. A., Newton, R. U., Wickham, R. B., Gotshalk, L. A., Duncan, N. D., Mazzetti, S. A., Gómez, A. L., Rubin, M. R., Nindl, B. C. and Häkkinen, K. (2001). The effect of heavy resistance exercise on the circadian rhythm of salivary testosterone in men. *European Journal of Applied Physiology* **84**: 13-18.

Kraemer, W. J., Marchitelli, L., Gordon, S. E., Harman, E., Dziados, J. E., Mello, R., Frykman, P., McCurry, D. and Fleck, S. J. (1990). Hormonal and growth factor responses to heavy resistance exercise protocols. *Journal of Applied Physiology* **69**: 1442-50.

Krieger, D. T., Allen, W., Rizzo, F. and Krieger, H. P. (1971). Characterization of the normal temporal pattern of plasma corticosteroid levels. *Journal of Clinical Endocrinology & Metabolism* **32**: 266-284.

Kroboth, P. D., Salek, F. S., Stone, R. A., Bertz, R. J. and Kroboth, F. J. (1999). Alprazolam increases dehydroepiandrosterone concentrations. *Journal of Clinical Psychopharmacology* **19**: 114-124.

Kruve, A., Herodes, K. and Leito, I. (2010). Optimisation of electrospray interface and quadrupole ion trap mass spectrometer parameters in pesticide liquid chromatography/electrospray ionisation mass spectrometry analysis. *Rapid Communications in Mass Spectrometry* **24**: 919-926.

Kushnir, M. M., Blamires, T., Rockwood, A. L., Roberts, W. L., Yue, B., Erdogan, E., Bunker, A. M. and Meikle, A. W. (2010). Liquid chromatography-tandem mass spectrometry assay for androstenedione, dehydroepiandrosterone, and testosterone with pediatric and adult reference intervals. *Clinical Chemistry* **56**: 1138-1147.

Kushnir, M. M., Rockwood, A. L., Roberts, W. L., Pattison, E. G., Bunker, A. M., Fitzgerald, R. L. and Meikle, A. W. (2006). Performance characteristics of a novel tandem mass spectrometry assay for serum testosterone. *Clinical Chemistry* **52**: 120-128.

Kushnir, M. M., Rockwood, A. L., Roberts, W. L., Yue, B., Bergquist, J. and Meikle, A. W. (2011). Liquid chromatography tandem mass spectrometry for analysis of steroids in clinical laboratories. *Clinical Biochemistry* **44**: 77-88.

Kutsukake, N., Ikeda, K., Honma, S., Teramoto, M., Mori, Y., Hayasaka, I., Yamamoto, R., Ishida, T., Yoshikawa, Y. and Hasegawa, T. (2009). Validation of salivary cortisol and testosterone assays in chimpanzees by liquid chromatography-tandem mass spectrometry. *American Journal of Primatology* **71**: 696-706.

Lac, G., Lac, N. and Robert, A. (1993). Steroid assays in saliva: A method to detect plasmatic contaminations. *Archives of Physiology and Biochemistry* **101**: 257-262.

Lambert, W. E. (2004). Pitfalls in LC-MS(MS) analysis. *TIAFT Bulletin* **24**: 59–61.

Landon, J. and Moffat, A. C. (1976). The radioimmunoassay of drugs. a review. *Analyst* **101**: 225-43.

Lansley, K. E., Dimenna, F. J., Bailey, S. J. and Jones, A. M. (2011). A new method to normalise exercise intensity. *International Journal of Sports Medicine* **32**: 535-541.

LaserChrom. (2011). "Laserchrom HPLC guide." Retrieved August 2011, from http://www.laserchrom.co.uk/LaserchromHPLC-pHBufferGuide.htm.

Lazarus, R. S. (1966). *Psychological stress and the coping process*. New York, McGraw-Hill.

Lazarus, R. S. and Cohen, J. B. (1977). Environmental stress. *Human behaviour and the environment: Current theory and research*. I. Attman and J. F. Wohlwill. New York, Plenum.

Le Panse, B., Vibarel-Rebot, N., Parage, G., Albrings, D., Amiot, V., De Ceaurriz, J. and Collomp, K. (2010). Cortisol, dhea, and testosterone concentrations in saliva in response to an international powerlifting competition. *Stress* **13**: 528-32.

Lee, S., Kwon, S., Shin, H. J., Lim, H. S., Singh, R. J., Lee, K. R. and Kim, Y. J. (2010). Simultaneous quantitative analysis of salivary cortisol and cortisone in Korean adults using LC-MS/MS. *Biochemistry and Molecular Biology Reports* **43**: 506-11.

Legrand, C., Dousset, B., Tronel, H., Belleville, F. and Nabet, P. (1995). Measurement of plasma testosterone by gas chromatography-negative-ion mass spectrometry using pentafluoropropionic derivatives. *Journal of Chromatography B* **663**: 187-192.

Lehmann, M. J., Lormes, W., Opitz-Gress, A., Steinacker, J. M., Netzer, N., Foster, C. and Gastmann, U. (1997). Training and overtraining: An overview and experimental results in endurance sports. *Journal of Sports Medicine and Physical Fitness* **31**: 7-17.

Lehmann, M., Foster, C., Netzer, N., Lormes, W., Steinacker, J. M., Lui, Y., Opitz-Gress, A. and Gastmann, U. (1998). Physiological responses to short and long term overtraining in endurance athletes. *Overtraining in sport*. R. B. Kreider, A. C. Fry and M. L. O'Toole. Champaign, ILL, Human Kinetics**:** 19-46.

Lehotay, S. J., de Kok, A., Hiemstra, M. and Van Bodegraven, P. (2005). Validation of a fast and easy method for the determination of residues from 229 pesticides in fruits and vegetables using gas and liquid chromatography and mass spectrometric detection. *Journal of Association of Official Analytical Chemists International* **88**: 595-614.

Leinonen, A., Kuuranne, T., Kotiaho, T. and Kostiainen, R. (2004). Screening of free 17-alkyl-substituted anabolic steroids in human urine by liquid chromatography electrospray ionisation tandem mass spectrometry. *Steroids* **69**: 101-109.

Levin, S. (2011, August 2011). Detection in hplc - qualification. Retrieved September, 2011, from www.forumsci.co.il/HPLC/Detection_qualifiction05.pdf.

Li, D., White, A. and Pudek, M. (2008). Liquid chromatography-tandem mass spectrometry analysis of salivary free cortisol: Validation and clinical application. *Clinical Biochemistry* **41**: 1273-1274.

Liang, H. R., Foltz, R. L., Meng, M. and Bennett, P. (2003). Ionisation enhancement in atmospheric pressure chemical ionisation and suppression in electrospray ionisation between target drugs and stable-isotope-labelled internal standards in quantitative liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **17**: 2815-2821.

Licea-Perez, H., Wang, S., Szapacs, M. E. and Yang, E. (2008). Development of a highly sensitive and selective uplc/ms/ms method for the simultaneous determination of testosterone and 5-dihydrotestosterone in human serum to support testosterone replacement therapy for hypogonadism. *Steroids* **73**: 601-610.

Liening, S. H., Stanton, S. J., Saini, E. K. and Schultheiss, O. C. (2010). Salivary testosterone, cortisol, and progesterone: two-week stability, interhormone correlations, and effects of time of day, menstrual cycle, and oral contraceptive use on steroid hormone levels. *Physiology and Behaviour* **99**: 8-16.

Linnamo, V., Pakarinen, A., Komi, P. V., Kraemer, W. J. and Hakkinen, K. (2005). Acute hormonal responses to submaximal and maximal heavy resistance and explosive exercises in men and women. *Journal of Strength and Conditioning Research* **19**: 566-71.

Ma, Y.-C. and Kim, H.-Y. (1997). Determination of steroids by liquid chromatography/mass spectrometry. *Journal of the American Society for Mass Spectrometry* **8**: 1010-1020.

Mackinnon, L. T. and Jenkins, D. G. (1993). Decreased salivary immunoglobulins after intense interval exercise before and after training. *Medicine & Science in Sports & Exercise* **25**: 678-683.

Maestu, J., Jurimae, J. and Jurimae, T. (2005). Hormonal response to maximal rowing before and after heavy increase in training volume in highly trained male rowers. *Journal of Sports Medicine and Physical Fitness* **45**: 121-6.

Magni, F., Pereira, S., Leoni, M., Grisenti, G. and Galli Kienle, M. (2001). Quantitation of cyclosporin A in whole blood by liquid chromatography/stable isotope dilution electrospray ionisation mass spectrometry. *Journal of Mass Spectrometry* **36**: 670-676.

Magnisali, P., Dracopoulou, M., Mataragas, M., Dacou-Voutetakis, A. and Moutsatsou, P. (2008). Routine method for the simultaneous quantification of 17[alpha]-hydroxyprogesterone, testosterone, dehydroepiandrosterone, androstenedione, cortisol, and pregnenolone in human serum of neonates using gas chromatography-mass spectrometry. *Journal of Chromatography A* **1206**: 166-177.

Majors, R. E. and Macdonald, F. R. (1973). Practical implications of modern liquid chromatographic column performance. *Journal of Chromatography A* **83**: 169-179.

Malamud, D. and Tabak, L. (1993). Saliva as a diagnostic fluid. *Annals of the New York Academy of Sciences* **694**: 1-348.

Mallet, C. R., Lu, Z. and Mazzeo, J. R. (2004). A study of ion suppression effects in electrospray ionisation from mobile phase additives and solid-phase extracts. *Rapid Communications in Mass Spectrometry* **18**: 49-58.

Marinelli, M., Sergio Roi, G., Giacometti, M., Bonini, P. and Banfi, G. (1994). Cortisol, testosterone, and free testosterone in athletes performing a marathon at 4,000 m altitude. *Hormone Research in Paediatrics* **41**: 225-229.

Marmur, A. (2000). Dissolution and self-assembly: the solvophobic/hydrophobic effect. *Journal of the American Chemical Society* **122**: 2120-2121.

Martin, W. H., Dalsky, G. P., Hurley, B. F., Matthews, D. E., Bier, D. M., Hagberg, J. M., Rogers, M. A., King, D. S. and Holloszy, J. O. (1993). Effect of endurance training on plasma free fatty acid turnover and oxidation during exercise. *American Journal of Physiology - Endocrinology And Metabolism* **265**: E708-E714.

Matsui, F., Koh, E., Yamamoto, K., Sugimoto, K., Sin, H. S., Maeda, Y., Honma, S. and Namiki, M. (2009). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for simultaneous measurement of salivary testosterone and cortisol in healthy men for utilization in the diagnosis of late-onset hypogonadism in males. *Endocrine Journal* **56**: 1083-93.

Mauras, N., Blizzard, R. M., Link, K., Johnson, M. L., Rogol, A. D. and Veldhuis, J. D. (1987). Augmentation of growth hormone secretion during puberty: Evidence for a pulse amplitude-modulated phenomenon. *Journal of Clinical Endocrinology and Metabolism* **64**: 596-601.

McCaulley, G. O., McBride, J. M., Cormie, P., Hudson, M. B., Nuzzo, J. L., Quindry, J. C. and Travis Triplett, N. (2009). Acute hormonal and neuromuscular responses to hypertrophy, strength and power type resistance exercise. *European Journal of Applied Physiology* **105**: 695-704.

McMillan, J. L., Stone, M. H., Sartin, J., Keith, R., Marples, D., Brown, C. and Lewis, R. D. (1993). 20-hour physiological responses to a single weight-training session. *Journal of Strength and Conditioning Research* **7**: 9-21.

McMurray, R. G. and Hackney, A. C. (2000). Endocrine responses to exercise and training. *Exercise and sport science*. W. E. Garrett and D. T. Kirkendall. Philidelphia, PA, Lippincott, Williams and Wilkins.

MedlinePlus. (2010a, June 2011). Testosterone. Retrieved June, 2011, from http://www.nlm.nih.gov/medlineplus/ency/article/003707.htm.

MedlinePlus. (2010b). Cortisol. Retrieved June, 2011, from http://www.nlm.nih.gov/medlineplus/ency/article/003703.htm.

Meeusen, R., Nederhof, E., Buyse, L., Roelands, B., de Schutter, G. and Piacentini, M. F. (2010). Diagnosing overtraining in athletes using the two-bout exercise protocol. *British Journal of Sports Medicine* **44**: 642-8.

Meeusen, R., Piacentini, M. F., Busschaert, B., Buyse, L., De Schutter, G. and Stray-Gundersen, J. (2004). Hormonal responses in athletes: The use of a two bout exercise protocol to detect subtle differences in (over)training status. *European Journal of Applied Physiology* **91**: 140-6.

Miller, K. K., Rosner, W., Lee, H., Hier, J., Sesmilo, G., Schoenfeld, D., Neubauer, G. and Klibanski, A. (2004). Measurement of free testosterone in normal women and women with androgen deficiency: Comparison of methods. *Journal of Clinical Endocrinology & Metabolism* **89**: 525-533.

Miller, W. L. (1988). Molecular biology of steroid hormone synthesis. *Endocrine Reviews* **9**: 295-318.

Moal, V., Mathieu, E., Reynier, P., Malthièry, Y. and Gallois, Y. (2007). Low serum testosterone assayed by liquid chromatography-tandem mass spectrometry. Comparison with five immunoassay techniques. *Clinica Chimica Acta* **386**: 12-19.

Monks, D. A., Kopachik, W., Breedlove, S. M. and Jordan, C. L. (2006). Anabolic responsiveness of skeletal muscles correlates with androgen receptor protein but not mrna. *Canadian Journal of Physiology and Pharmacology* **84**: 273-7.

Moreira, A., Arsati, F., de Oliveira Lima Arsati, Y. B., da Silva, D. A. and de Araujo, V. C. (2009). Salivary cortisol in top-level professional soccer players. *European Journal of Applied Physiology* **106**: 25-30.

Moreira, A., Arsati, F., Lima-Arsati, Y. B., Franchini, E. and De Araujo, V. C. (2010). Effect of a kickboxing match on salivary cortisol and immunoglobulin a. *Perception and Motor Skills* **111**: 158-66.

Morley, J. E., Kaiser, F. E., Perry, H. M., 3rd, Patrick, P., Morley, P. M., Stauber, P. M., Vellas, B., Baumgartner, R. N. and Garry, P. J. (1997). Longitudinal changes in testosterone, luteinizing hormone, and follicle-stimulating hormone in healthy older men. *Metabolism* **46**: 410-3.

Muller, C., Schafer, P., Stortzel, M., Vogt, S. and Weinmann, W. (2002). Ion suppression effects in liquid chromatography-electrospray-ionisation transport-region collision induced dissociation mass spectrometry with different serum extraction methods for systematic toxicological analysis with mass spectra libraries. *Journal of Chromatography B* **15**: 47-52.

Murata, H., Takao, T., Shimonishi, Y. and Matsuo, T. (1994). Optimisation of skimmer voltages of an electrospray ion source coupled with a magnetic sector instrument. *Rapid Communications in Mass Spectrometry* **8**: 205-210.

Nelson, E. A., Palombo, E. A. and Knowles, S. R. (2008). Comparison of evaporation techniques for the preparation of salivary cortisol for analysis by liquid chromatography-electrospray tandem mass spectrometry. *Clinical Biochemistry* **41**: 1413-1416.

Nindl, B. C., Kraemer, W. J., Deaver, D. R., Peters, J. L., Marx, J. O., Heckman, J. T. and Loomis, G. A. (2001). LH secretion and testosterone concentrations are blunted after resistance exercise in men. *Journal of Applied Physiology* **91**: 1251-1258.

Nieman, D. C. (1994). Exercise, upper respiratory tract infection, and the immune system. *Medicine & Science in Sports & Exercise* **26**: 128-139.

Norman, A. W. and Litwack, G. (1997). *Hormones*. San Diego, Academic.

Null, A. P., Nepomuceno, A. I. and Muddiman, D. C. (2003). Implications of hydrophobicity and free energy of solvation for characterization of nucleic acids by electrospray ionisation mass spectrometry. *Analytical Chemistry* **75**: 1331-1339.

Nunes, J. A., Crewther, B. T., Ugrinowitsch, C., Tricoli, V., Viveiros, L., De Rose, D., Jr. and Aoki, M. S. (2011). Salivary hormone and immune responses to three resistance exercise schemes in elite female athletes. *Journal of Strength and Conditioning Research* **25**: 2322-2327.

O'Connor, P. J. and Corrigan, D., L (1987). Influence of short-term cycling on salivary cortisol levels. *Medicine & Science in Sports & Exercise* **19**: 224-228.

Owen, L. J., Haslam, S., Adaway, J. E., Wood, P., Glenn, C. and Keevil, B. G. (2010). A simplified liquid chromatography tandem mass spectrometry assay, using on-line solid-phase extraction, for the quantitation of cortisol in saliva and comparison with a routine delfia method. *Annals of Clinical Biochemistry* **47**: 131-136.

Paccotti, P., Minetto, M., terzolo, M., Ventura, M., Ganzit, G. P., Borrione, P., Termine, A. and Angeli, A. (2005). Effects of high intensity isokinetic exercise on salivary cortisol in athletes with different training schedules: Relationships to serum cortisol and lactate. *International Journal of Sports Medicine* **26**: 747-755.

Passelergue, P. and Lac, G. (1999). Saliva cortisol, testosterone and t/c ratio variations during a wrestling competition and during the post-competitive recovery period. *International Journal of Sports Medicine* **20**: 109-13.

Patel, K. N., Patel, J. K., Rajput, G. C. and Patel, H. A. (2010). Ultra performance liquid chromatography (uplc): An introduction and its application. *PharmExpt* **1**.

Payne, A. H. and Hales, D. B. (2004). Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocrine Reviews* **25**: 947-970.

Perogamvros, I., Owen, L. J., Keevil, B. G., Brabant, G. and Trainer, P. J. (2010). Measurement of salivary cortisol with liquid chromatography-tandem mass spectrometry in patients undergoing dynamic endocrine testing. *Clinical Endocrinology* **72**: 17-21.

Petibois, C., Cazorla, G., Poortmans, J. R., eacute and ris, G. (2002). Biochemical aspects of overtraining in endurance sports: A review. *Sports Medicine* **32**: 867-878.

Petraglia, F., Barletta, C., Facchinetti, F., Spinazzola, F., Monzani, A., Scavo, D. and Genazzani, A. R. (1988). Response of circulating adrenocorticotropin, beta-endorphin, beta-lipotropin and cortisol to athletic competition. *Acta Endocrinologica* **118**: 332-6.

Phenomenex (2008). Explore Luna PFP discover selectivity.

Phenomenex (2010). Column care guide.

Pilardeau, P., Richalet, J. P., Bouissou, P., Vaysse, J., Larmignat, P. and Boom, A. (1990). Saliva flow and composition in humans exposed to acute altitude hypoxia. *European Journal of Applied Physiology and Occupational Physiology* **59**: 450-453.

Poll, E.-M., Kreitschmann-Andermahr, I., Langejuergen, Y., Stanzel, S., Gilsbach, J. M., Gressner, A. and Yagmur, E. (2007). Saliva collection method affects predictability of serum cortisol. *Clinica Chimica Acta* **382**: 15-19.

Poole, C. F. (2003). New trends in solid-phase extraction. *Trends in Analytical Chemistry* **22**: 362-373.

Pruessner, J. C., Hellhammer, D. H. and Kirschbaum, C. (1999). Burnout, perceived stress, and cortisol responses to awakening. *Psychosomatic medicine* **61**: 197-204.

Pruessner, J. C., Wolf, O. T., Hellhammer, D. H., Buske-Kirschbaum, A., von Auer, K., Jobst, S., Kaspers, F. and Kirschbaum, C. (1997). Free cortisol levels after awakening: a reliable biological marker for the assessment of adrenocortical activity. *Life Sciences* **61**: 2539-2549.

Pucsok, J. M., Gyore, I., Hollosi, I., Soos, E., Ali Ghasemi, N. R. and Frenkl, R. (2005). Urine steroid profile of judo competitors affected by acute physical exercises. *Journal of Chromatographic Science* **43**: 438-40.

Raastad, T., Bjøro, T. and Hallén, J. (2000). Hormonal responses to high- and moderate-intensity strength exercise. *European Journal of Applied Physiology* **82**: 121-128.

Raff, H., Homar, P. J. and Burns, E. A. (2002). Comparison of two methods for measuring salivary cortisol. *Clinical Chemistry* **48**: 207-208.

Rayleigh, L. (1882). On the equilibrium of liquid conducting masses charged with electricity. *Philosophical Magazine* **14**: 184–186.

Resko, J. A. and Eik-Nes, K. B. (1966). Diurnal testosterone levels in peripheral plasma of human male subjects. *The Journal of Clinical Endocrinology & Metabolism* **26**: 573-576.

Rose, R. M., Kreuz, L. E., Holaday, J. W., Sulak, K. J. and Johnson, C. E. (1972). Diurnal variation of plasma testosterone and cortisol. *Journal of Endocrinology* **54**: 177-178.

Rosner, W. (1990). The functions of corticosteroid-binding globulin and sex hormone-binding globulin: Recent advances. *Endocrine Reviews* **11**: 80-91.

Rosner, W., Auchus, R. J., Azziz, R., Sluss, P. M. and Raff, H. (2007). Utility, limitations, and pitfalls in measuring testosterone: An endocrine society position statement. *Journal of Clinical Endocrinology and Metabolism* **92**: 405-413.

Rowbottom, D. G., Keast, D., Garcia-Webb, P. and Morton, A. R. (1995). Serum free cortisol responses to a standard exercise test among elite triathletes. *Australian Journal of Science and Medicine in Sport* **27**: 103-7.

Rudolph, D. L. and McAuley, E. (1998). Cortisol and affective responses to exercise. *Journal of Sports Sciences* **16**: 121-8.

Salimetrics (2011). Salivary cortisol enzyme immunoassay kit, revised march 2011.

Salway, J. G. (2006). *Medical biochemistry at a glance*. Oxford, Blackwell.

Santa, T., Al-Dirbashi, O. Y., Ichibangase, T., Fukushima, T., Rashed, M. S., Funatsu, T. and Imai, K. (2007). Synthesis of benzofurazan derivatization reagents for carboxylic acids in liquid chromatography/electrospray ionisation–tandem mass spectrometry. *Biomedical Chromatography* **21**: 1207-1213.

Santtila, M., Kyrolainen, H. and Hakkinen, K. (2009). Serum hormones in soldiers after basic training: Effect of added strength or endurance regimens. *Aviation, Space and Environmental Medicine* **80**: 615-20.

Sari-Sarraf, V., Reilly, T., Doran, D. A. and Atkinson, G. (2007). The effects of single and repeated bouts of soccer-specific exercise on salivary IgA. *Archives of Oral Biology* **52**: 526-32.

Saudan, C., Baume, N., Robinson, N., Avois, L., Mangin, P. and Saugy, M. (2006). Testosterone and doping control. *British Journal of Sports Medicine* **40**: i21-i24.

Schmikli, S. L., Brink, M. S., de Vries, W. R. and Backx, F. J. (2011). Can we detect non-functional overreaching in young elite soccer players and middle-long distance runners using field performance tests? *British Journal of Sports Medicine* **45**: 631-6.

Schulz, P., Kirschbaum, C.and Hellhammer, D. (1998). Increased free cortisol secretion after awakening in chronically stressed individuals due to work overload. *Stress and Health* **14**: 91-97.

Schwartz, E. B. and Granger, D. A. (2004). Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clinical Chemistry* **50**: 654-656.

Shackleton, C. H., Chuang, H., Kim, J., de la Torre, X. and Segura, J. (1997). Electrospray mass spectrometry of testosterone esters: Potential for use in doping control. *Steroids* **62**: 523-9.

Shen, Y., Zhao, R., Berger, S. J., Anderson, G. A., Rodriguez, N. and Smith, R. D. (2002). High-efficiency nanoscale liquid chromatography coupled on-line with mass spectrometry using nanoelectrospray ionisation for proteomics. *Analytical Chemistry* **74**: 4235-4249.

Shibasaki, H., Nakayama, H., Furuta, T., Kasuya, Y., Tsuchiya, M., Soejima, A., Yamada, A. and Nagasawa, T. (2008). Simultaneous determination of prednisolone, prednisone, cortisol, and cortisone in plasma by GC-MS: estimating unbound prednisolone concentration in patients with nephrotic syndrome during oral prednisolone therapy. *Journal of Chromatography B* **870**: 164-169.

Shibayama, Y., Higashi, T., Shimada, K., Kashimada, K.-I., Onishi, T., Ono, M., Miyai, K. and Mizutani, S. (2008). Liquid chromatography-tandem mass spectrometric method for determination of salivary 17[alpha]-hydroxyprogesterone: A noninvasive tool for evaluating efficacy of hormone replacement therapy in congenital adrenal hyperplasia. *Journal of Chromatography B* **867**: 49-56.

Shibayama, Y., Higashi, T., Shimada, K., Odani, A., Mizokami, A., Konaka, H., Koh, E. and Namiki, M. (2009). Simultaneous determination of salivary testosterone and dehydroepiandrosterone using LC-MS/MS: Method development and evaluation of applicability for diagnosis and medication for late-onset hypogonadism. *Journal of Chromatography B* **877**: 2615-23.

Shimazdu. (2010). "Switching the mobile phase from acetonitrile to methanol " Retrieved September, 2011, from http://www.shimadzu.com/an/hplc/support/faq/acetonitrile/2-1.html.

Shiraishi, S., Lee, P. W. N., Leung, A., Goh, V. H. H., Swerdloff, R. S. and Wang, C. (2008). Simultaneous measurement of serum testosterone and dihydrotestosterone by liquid chromatography-tandem mass spectrometry. *Clinical Chemistry* **54**: 1855-1863.

Simon, D., Preziosi, P., Barrett-Connor, E., Roger, M., Saint-Paul, M., Nahoul, K. and Papoz, L. (1992). The influence of aging on plasma sex hormones in men: The telecom study. *Americal Journal of Epidemiology* **135**: 783-91.

Singh, R. J. (2008). Validation of a high throughput method for serum/plasma testosterone using liquid chromatography tandem mass spectrometry (LC-MS/MS). *Steroids* **73**: 1339-1344.

Smilios, I., Pilianidis, T., Karamouzis, M. and Tokmakidis, S. P. (2003). Hormonal responses after various resistance exercise protocols. *Medicine & Science in Sports & Exercise* **35**: 644-54.

Smith, J. N., Flagan, R. C. and Beauchamp, J. L. (2002). Droplet evaporation and discharge dynamics in electrospray ionisation *The Journal of Physical Chemistry A* **106**: 9957-9967.

Soler, C., MaÃ±es, J. and PicÃ³, Y. (2005). Routine application using single quadrupole liquid chromatography mass spectrometry to pesticides analysis in citrus fruits. *Journal of Chromatography A* **1088**: 224-233.

Stanczyk, F. Z., Lee, J. S. and Santen, R. J. (2007). Standardisation of steroid hormone assays: Why, how, and when? *Cancer Epidemiology Biomarkers and Prevention* **16**: 1713-1719.

Steensberg, A., Van Hall, G., Keller, C., Osada, T., Schjerling, P., Pedersen, B. K., Saltin, B. and Febbraio, M. A. (2002). Muscle glycogen content and glucose uptake during exercise in humans: Influence of prior exercise and dietary manipulation. *Journal of Physiology* **15**: 273-281.

Sterner, J. L., Johnston, M. V., Nicol, G. R. and Ridge, D. P. (2000). Signal suppression in electrospray ionisation fourier transform mass spectrometry of multi-component samples. *Journal of Mass Spectrometry* **35**: 385-391.

Stone, M. H. and Fry, A. C. (1997). Increased training volume in strength\power athletes. *Overtraining in sport*. R. B. Kreider, A. C. Fry and M. L. O'Toole. Champaign, Il, Human Kinetics**:** 87-106.

Stuempfle, K. J., Nindl, B. C. and Kamimori, G. H. (2010). Stress hormone responses to an ultraendurance race in the cold. *Wilderness and Environmental Medicine* **21**: 22-7.

Supelco (1998). Guide to solid phase extraction. *Bulletin* **910**.

Sutton, J. R., Coleman, M. J., Casey, J. and Lazarus, L. (1973). Androgen responses during physical exercise. *British Medical Journal* **1**: 520-522.

Szulc, P., Munoz, F., Claustrat, B., Garnero, P., Marchand, F., Duboeuf, F. and Delmas, P. D. (2001). Bioavailable estradiol may be an important determinant of osteoporosis in men: The minos study. *Journal of Clinical Endocrinology and Metabolism* **86**: 192-9.

Taieb, J., Mathian, B., Millot, F., Patricot, M.-C., Mathieu, E., Queyrel, N., Lacroix, I., Somma-Delpero, C. and Boudou, P. (2003). Testosterone measured by 10 immunoassays and by isotope-dilution gas chromatography-mass spectrometry in sera from 116 men, women, and children. *Clinical Chemistry* **49**: 1381-1395.

Tang, L. and Kebarle, P. (1993). Dependence of ion intensity in electrospray mass spectrometry on the concentration of the analytes in the electrosprayed solution. *Analytical Chemistry* **65**: 3654-3668.

Tanskanen, M. M., Kyrolainen, H., Uusitalo, A. L., Huovinen, J., Nissila, J., Kinnunen, H., Atalay, M. and Hakkinen, K. (2011). Serum sex hormone-binding globulin and cortisol concentrations are associated with overreaching during strenuous military training. *Journal of Strength and Conditioning Research* **25**: 787-97.

Tarnopolsky, L. J., Macdougall, J. D., Atkinson, S. A., Tarnopolsky, M. A. and Sutton, J. R. (1990). Gender differences in substrate for endurance exercise. *Journal of Applied Physiology* **68**: 302-308.

Taylor, G. (1964). Disintegration of water drops in an electric field. *Proceedings of the Royal Society A: Mathematical, Physical and Engineering Sciences* **280**: 383-397.

Taylor, P. J. (2005). Matrix effects: The achilles heel of quantitative high-performance liquid chromatography electrospray tandem mass spectrometry. *Clinical Biochemistry* **38**: 328-334.

Taylor, P. J., van Rosendal, S. P., Coombes, J. S., Gordon, R. D. and Stowasser, M. (2010). Simultaneous measurement of aldosterone and cortisol by high-performance liquid chromatography-tandem mass spectrometry: Application to dehydration-rehydration studies. *Journal of Chromatography B* **878**: 1195-8.

Tennekoon, K. H. and Karunanayake, E. H. (1993). Serum FSH, LH, and testosterone concentrations in presumably fertile men: Effect of age. *International Journal of Fertility* **38**: 108-12.

Thermo (2004). *HPLC analysis of biomolecules technical guide: Successful separation of peptides, proteins and other biomolecules*.

Thevis, M., Bommerich, U., Opfermann, G. and Schänzer, W. (2005). Characterisation of chemically modified steroids for doping control purposes by electrospray ionisation tandem mass spectrometry. *Journal of Mass Spectrometry* **40**: 494-502.

Thevis, M. and Schanzer, W. (2007). Current role of LC-MS(/MS) in doping control. *Analytical and Bioanalytical Chemistry* **388**: 1351-8.

Thomas, N. E., Leyshon, A., Hughes, M. G., Davies, B., Graham, M. and Baker, J. S. (2009). The effect of anaerobic exercise on salivary cortisol, testosterone and immunoglobulin (A) in boys aged 15-16 years. *European Journal of Applied Physiology* **107**: 455-61.

Thomas, N. E., Leyshon, A., Hughes, M. G., Jasper, M. A., Davies, B., Graham, M. R., Bulloch, J. M. and Baker, J. S. (2010). Concentrations of salivary testosterone, cortisol, and immunoglobulin A after supra-maximal exercise in female adolescents. *Journal of Sports Sciences* **28**: 1361-8.

Timon, R., Olcina, G., Munoz, D., Maynar, J. I., Caballero, M. J. and Maynar, M. (2008). Determination of urine steroid profile in untrained men to evaluate recovery after a strength training session. *Journal of Strength and Conditioning Research* **22**: 1087-93.

Tremblay, M. S., Copeland, J. L. and Van Helder, W. (2005). Influence of exercise duration on post-exercise steroid hormone responses in trained males. *European Journal of Applied Physiology* **94**: 505-13.

Tuck, M. K., Chan, D. W., Chia, D., Godwin, A. K., Grizzle, W. E., Krueger, K. E., Rom, W., Sanda, M., Sorbara, L., Stass, S., Wang, W. and Brenner, D. E. (2008). Standard operating procedures for serum and plasma collection: early detection research network consensus statement standard operating procedure integration working group. *Journal of Proteome Research* **8**: 113-117.

Tullo, A. (2008). A solvent dries up. *Chemical & Engineering News* **86**: 27.

U.S., Department, of, Health, and, Human and Services (2001). Guidance for industry: Bioanalytical method validation.

Urhausen, A., Gabriel, H. and Kindermann, W. (1995). Blood hormones as markers of training stress and overtraining. *Sports Medicine* **20**: 251-76.

Urhausen, A. and Kindermann, W. (2002). Diagnosis of overtraining: what tools do we have? *Sports Medicine* **32**: 95-102.

van Deemter, J. J., Zuiderweg, F. J. and Klinkenberg, A. (1956). Longitudinal diffusion and resistance to mass transfer as causes of nonideality in chromatography. *Chemical Engineering Science* **5**: 271-289.

Van Uytfanghe, K., Stockl, D., Kaufman, J. M., Fiers, T., De Leenheer, A. and Thienpont, L. M. (2005). Validation of 5 routine assays for serum free testosterone with a candidate reference measurement procedure based on ultrafiltration and isotope dilution-gas chromatography-mass spectrometry. *Clinical Biochemistry* **38**: 253-61.

Vanhoenacker, G. and Sandra, P. (2006). Elevated temperature and temperature programming in conventional liquid chromatography – fundamentals and applications. *Journal of Separation Science* **29**: 1822-1835.

Vasankari, T. J., Kujala, U. M., Heinonen, O. J. and Huhtaniemi, I. T. (1993). Effects of endurance training on hormonal responses to prolonged physical exercise in males. *European Journal of Endocrinology* **129**: 109-113.

Vermeulen, A., Rubens, R. and Verdonck, L. (1972). Testosterone secretion and metabolism in male senescence. *Journal of Clinical Endocrinology and Metabolism* **34**: 730-5.

Vervoorn, C., Quist, A. M., Vermulst, L. J. M., Erich, W. B. M., de Vries, W. R. and Thijssen, J. H. H. (1991). The behaviour of the plasma free testosterone/cortisol ratio during a season of elite rowing training. *International Journal of Sports Medicine* **12**: 257,263.

Vesper, H. W. and Botelho, J. C. (2010). Standardisation of testosterone measurements in humans. *The Journal of Steroid Biochemistry and Molecular Biology* **121**: 513-519.

Vingren, J. L., Kraemer, W. J., Ratamess, N. A., Anderson, J. M., Volek, J. S. and Maresh, C. M. (2010). Testosterone physiology in resistance exercise and training: The up-stream regulatory elements. *Sports Medicine* **40**: 1037-53.

Vining, R., McGinley, R. and Symons, R. (1983). Hormones in saliva: mode of entry and consequent implications for clinical interpretation. *Clinical Chemistry* **29**: 1752-1756.

Viru, A., Hackney, A., Välja, E., Karelson, K., Janson, T. and Viru, M. (2001). Influence of prolonged continuous exercise on hormone responses to subsequent exercise in humans. *European Journal of Applied Physiology* **85**: 578-585.

Viru, A., Karelson, K. and Smirnova, T. (1992). Stability and variability in hormonal responses to prolonged exercise. *International Journal of Sports Medicine* **13**: 230-5.

Vittek, J., G, L. H. D., Gordon, G. G., Rappaport, S. C. and Southren, A. L. (1985). Direct radioimmunoassay (RIA) of salivary testosterone correlation with free and total serum testosterone. *Life Sciences* **37**: 711-716.

Vollestad, N. K. and Blom, P. C. S. (1985). Effect of varying exercise intensity on glycogen depletion in human muscle fibres. *Acta Physiologica Scandinavica* **125**: 395-405.

Vuorimaa, T., Ahotupa, M., Hakkinen, K. and Vasankari, T. (2008). Different hormonal response to continuous and intermittent exercise in middle-distance and marathon runners. *Scandinavian Journal of Medicine and Science in Sports* **18**: 565-72.

Walton, M. J., Anderson, R. A., Kicman, A. T., Elton, R. A., Ossowska, K. and Baird, D. T. (2007). A diurnal variation in testicular hormone production is maintained following gonadotrophin suppression in normal men. *Clinical Endocrinology* **66**: 123-9.

Wang, C., Catlin, D. H., Demers, L. M., Starcevic, B. and Swerdloff, R. S. (2004). Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography-tandem mass spectrometry. *Journal of Clinical Endocrinology and Metabolism* **89**: 534-43.

Wang, C., Plymate, S., Nieschlag, E. and Paulsen, C. (1981). Salivary testosterone in men: further evidence of a direct correlation with free serum testosterone. *Journal of Clinical Endocrinology and Metabolism* **53**: 1021-1024.

Wang, C., Shiraishi, S., Leung, A., Baravarian, S., Hull, L., Goh, V., Lee, P. W. N. and Swerdloff, R. S. (2008). Validation of a testosterone and dihydrotestosterone liquid chromatography tandem mass spectrometry assay: Interference and comparison with established methods. *Steroids* **73**: 1345-1352.

Wang, G. and Cole, R. B. (1997). Solution, gas phase and instrumental parameter influences on charge state distributions in electrospray ionisation mass spectrometry. *Electrospray ionisation mass spectrometry: Fundamentals, instrumentation and applications*. R. B. Cole. New York, John Wiley & Sons**:** 137-174

Waters (2011). Quattro micro - guide to tuning. Product information sheet 30184**:** 1-13.

Watson, T. J. and Sparkman, D. O. (2007). *Introduction to mass spectrometry*. Chichester, UK, John Wiley and Sons.

Weekes, N. Y., Lewis, R. S., Goto, S. G., Garrison-Jakel, J., Patel, F. and Lupien, S. (2008). The effect of an environmental stressor on gender differences on the awakening cortisol response. *Psychoneuroendocrinology* **33**: 766-772.

Weise, M., Eisenhofer, G. and Merke, D. P. (2002). Pubertal and gender-related changes in the sympathoadrenal system in healthy children. *Journal of Clinical Endocrinology and Metabolism* **87**: 5038-43.

Whembolua, G.-L. S., Granger, D. A., Singer, S., Kivlighan, K. T. and Marguin, J. A. (2006). Bacteria in the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Hormones and Behaviour* **49**: 478-483.

Wieling, J. (2002). LC-MS-MS experiences with internal standards. *Chromatographia* **55**: S107-S113.

Wilm, M. S. and Mann, M. (1994). Electrospray and Taylor-cone theory, dole's beam of macromolecules at last? *International Journal of Mass Spectrometry and Ion Processes* **136**: 167-180.

Wilson, J. D. (1998). *Williams textbook of endocrinology*. Philideliphia, Saunders.

Wolf, O. T., Schommer, N. C., Hellhammer, D. H., McEwen, B. S. and Kirschbaum, C. (2001). The relationship between stress induced cortisol levels and memory differs between men and women. *Psychoneuroendocrinology* **26**: 711-20.

Wust, S., Federenko, I., Hellhammer, D. H. and Kirschbaum, C. (2000). Genetic factors, perceived chronic stress, and the free cortisol response to awakening. *Psychoneuroendocrinology* **25**: 707-720.

Yalow, R. S. and Berson, S. A. (1959). Assay of plasma insulin in human subjects by immunological methods. *Nature* **184**: 1648-1649.

Yamashita, K., Miyashiro, Y., Maekubo, H., Okuyama, M., Honma, S., Takahashi, M. and Numazawa, M. (2009). Development of highly sensitive quantification method for testosterone and dihydrotestosterone in human serum and prostate tissue by liquid chromatography-electrospray ionisation tandem mass spectrometry. *Steroids* **74**: 920-926.

Yamashita, K., Takahashi, M., Tsukamoto, S., Numazawa, M., Okuyama, M. and Honma, S. (2007). Use of novel picolinoyl derivatization for simultaneous quantification of six corticosteroids by liquid chromatography-electrospray ionisation tandem mass spectrometry. *Journal of Chromatography A* **1173**: 120-128.

Yap, B. K., Kazlauskas, R., Elghazi, K., Johnston, G. A. R. and Weatherby, R. P. (1996). Profiling of urinary testosterone and luteinizing hormone in exercise-stressed male athletes, using gas chromatography-mass spectrometry and enzyme immunoassay techniques. *Journal of Chromatography B* **687**: 117-125.

Yarrow, J. F., Borsa, P. A., Borst, S. E., Sitren, H. S., Stevens, B. R. and White, L. J. (2007). Neuroendocrine responses to an acute bout of eccentric-enhanced resistance exercise. *Medicine & Science in Sports & Exercise* **39**: 941-7.

Yasuda, M., Honma, S., Furuya, K., Yoshii, T., Kamiyama, Y., Ide, H., Muto, S. and Horie, S. (2008). Diagnostic significance of salivary testosterone measurement revisited: Using liquid chromatography/mass spectrometry and enzyme-linked immunosorbent assay. *Journal of Men's Health* **5**: 56-63.

Yokokawa, A., Yamamoto, K., Omori, Y., Shibasaki, H., Shinohara, Y., Kasuya, Y. and Furuta, T. (2009). Simultaneous determination of androstenedione, 11[beta]-hydroxyandrostenedione, and testosterone in human plasma by stable isotope dilution mass spectrometry. *Journal of Chromatography B* **877**: 621-626.

Yoshida, T., Chida, M., Ichioka, M. and Suda, Y. (1987). Blood lactate parameters related to aerobic capacity and endurance performance. *European Journal of Applied Physiology and Occupational Physiology* **1**: 7-11.

Yost, R. A. and Enke, C. G. (1978). Selected ion fragmentation with a tandem quadrupole mass spectrometer. *Journal of the American Chemical Society* **100**: 2274-2275.

APPENDICES

Appendix A – Informed consent form

Participant Consent Form

Participant number.....

Title of Research:		
Investigator's name: Amy Tanner		
To be completed by the participant:		
1.	Have you read the information sheet about this study?	YES/NO
2.	Have you had an opportunity to ask questions and discuss this study?	YES/NO
3.	Have you received satisfactory answers to all your questions?	YES/NO
4.	Have you received enough information about this study?	YES/NO
5.	Do you understand that you are free to withdraw from this study:	
	• at any time?	YES/NO
	• without giving a reason for withdrawing?	YES/NO
	• without affecting your future with the University/studies/medical or nursing care?	YES/NO
6.	Do you agree to take part in this study?	YES/NO
Signed		Date
Name in block letters		
Signature of investigator		Date

This Project is Supervised by: Judith Allgrove
Contact Details (including telephone number and email address): **J.E.Allgrove@gre.ac.uk**

Appendix B – Health questionnaire

the UNIVERSITY *of* GREENWICH

CONFIDENTIAL

Pre-test Health & Physical Activity Questionnaire

Date: .....
Name: .....
D.O.B: .....
Tel. No: .....
E. Mail: .....

Sex: .....
Address: .....
.....
.....
.....
Postcode: .....

Please circle when appropriate

1. Do you, or have you ever smoked? Yes/No

   If yes please state the number/day ..... or when stopped .....

2. Do you drink alcohol regularly? Yes/No

   If yes how many units/week? (1/2 pint = 1 units) .....

3. Have you consulted your general practitioner with the last 3 months? Yes/No

   If yes please give details .....

4. Are you on any medication at present? Yes/No

   If yes please state which and for what .....

   .....

5. When was the last time you had a medical check-up? .....

6. Have you ever suffered from:-

Any heart condition	Yes/No
If yes please specify	
High blood-pressures (>140/90)	Yes/No
Fainting	Yes/No
Heart or chest pains	Yes/No
Anaemia	Yes/No
Family history of heart of vascular disease	Yes/No
If yes please specify	
High blood cholesterol (>5.2mmol/L) if known	Yes/No
Any blood condition	Yes/No
If yes please specify	
HIV, Hepatitis A, B or C, Venereal Disease, Haemophilia, Any other	
Respiratory problems (asthma, bronchitis, etc.)	Yes/No
If yes please specify	
Diabetes - NIDDM or IDDM (please circle)	Yes/No
Epilepsy	Yes/No
Cancer	Yes/No
If yes please specify	

7. Are you currently injured Yes/No

If yes please specify .....

8. Have you been ill within the last 3 weeks? Yes/No

If yes please specify .....

9. Have you ever “over-reached”, had overtraining syndrome or chronic fatigue Syndrome? Yes/No

If yes please specify .....

10. To your knowledge are there any health related reasons for not undergoing the tests that have been explained to you? Yes/No

    If yes please specify .....

11. How many times do you exercise every week .....

    Do you weight train? Yes/No

    If yes how many times per week?.....

    play games? Yes/No

    If yes how many times per week and what games?.....

    swim, run or cycle? Yes/No

If yes:

what exercise? .....

how many times per week? .....

for how long? .....

13. Are you out of breath during exercise; (always?) 10.....5.....0 (never)

14. Are there any other relevant factors? .....

.....

15. Height (metres) ..... Weight (kg) .....

Signature of participant: ..... **Date** .....

Signature of researcher: ..... **Date** .....

RISK ASSESSMENT (ACSM Guidelines)

No. of Cardiopulmonary signs / symptoms .....

No of Risk Factors .....

Recommendation .....

Assessors signature .....

Appendix C – Participant information sheet resting study

the UNIVERSITY of GREENWICH

Research Information Sheet for Participants Involved in the Study: Detection, measurement and validation of stress biomarkers in saliva with mass spectrometry

Firstly thank you for agreeing to take part in this research project. I hope you find the information given here useful and adequately informative for your participation in the project. The research aims to validate a method to measure stress hormones (cortisol, testosterone and DHEA) in saliva using mass spectrometry.

Procedures
Prior to all testing you will arrive in the laboratory at 9am and complete the health questionnaire and informed consent form. You will be required to attend the Physiology Laboratory or Laboratory 148, Grenville Building, University of Greenwich at Medway, Central Avenue, Chatham Maritime, Kent ME4 4TB on one occasion.

Measurements
A three minute saliva sample will be collected in a tube via passive drool

Requirements
Participants should refrain from eating one hour prior to arriving at the laboratory and should refrain from drinking five minutes before the saliva sample so as not to dilute the sample.
Possible Risks/Discomforts
The risks involved in the study are minimal. Prior to the study, you will have filled out a health questionnaire to assess your suitability for participation in the study. Participants should seek advice from their GP if the levels are outside the expected normal range.

You are free to withdraw from the study at any time.

Benefits
The study will provide you with information about the levels of stress hormones in your saliva i.e. cortisol. testosterone, DHEA. You will be advised to seek advice from their GP if the levels are outside the expected normal range
Confidentiality
All data obtained will be dealt with in a confidential manner in line with the Data Protection Act. Data will be held for no more than two years before being destroyed.

I look forward to seeing you, if there are any problems please do not hesitate to call me on one of the numbers below:

Investigator: Amy Tanner
Email:
A.V.Tanner@gre.ac.uk

Supervisor: Dr Judith Allgrove
Email: J.E.Allgrove@gre.ac.uk

Appendix D - participant information sheet exercise study

Research Information Sheet for Participants Involved in the Study: Effect of three different training sessions on salivary and plasma stress hormones and immune response in competitive runners

Firstly thank you for your interest in this research project. I hope you find the information given here useful and adequately informative for your participation in the project. The research aims to monitor salivary cortisol, testosterone and immunoglobulin A (IgA) levels before, during and after three simulated training sessions and to compare the levels of cortisol and testosterone with those in simultaneous plasma samples in order to see if there is a correlation between plasma and salivary levels of these hormones.

Procedures
Prior to all testing you will complete the health questionnaire and informed consent form.

Measurements
The study will involve five visits to the laboratory:

Visit 1: VO2max assessment and study familiarisation i.e. getting used to changing the speed on the treadmill to simulate training and giving saliva samples.

Visits 2, 3, 4 and 5 either one of three training protocols (below) or a resting trial

Interval training
6 x 4mins of running at 90% VO2max with recoveries of 2 mins between repetitions at 30% VO2max

Tempo run
30 minutes of running at 70% VO2max (or lactate threshold)

Circuit training
10 exercise stations performing 3x30secs with 30secs recovery for each exercise, total duration is 30 mins

Resting trial
Participant sits down and rests of the duration of the trial (30 mins)

Blood measures via cannulation will be taken pre exercise, post exercise (30 mins) and at 15, 30 and 60 mins post exercise.

Saliva samples will be collected at the same time intervals, a 3 mins stimulated sample will be collected for the analysis of cortisol and testosterone and a 2 mins unstimulated sample for IgA.

You will also be required to complete an illness incidence questionnaire and stress questionnaire prior to each trial

Requirements
Participants should refrain from eating two hours prior to entering the laboratory and refrain from drinking water 5 minutes before producing the first sample

Possible Risks/Discomforts
The risks involved in the study are minimal. Prior to the study you will have filled out a health questionnaire to assess your suitability for participation in the study. Blood sampling via cannulation can be slightly uncomfortable and cause minor bruising but good practice by qualified personnel minimises this risk. Participants should seek advice from their GP if the levels are outside the expected normal range.

You are free to withdraw from the study at any time.

Benefits
You will gain a reading of your VO2max level which is an indicator of fitness. You will also gain results of your resting salivary and plasma cortisol and testosterone levels and the effect of the three training sessions on these levels, which could be useful when planning training sessions. Similarly, resting and exercise salivary IgA will be measured and this is an indication of basic immune status and the levels can be linked with potential illness incidence.

Confidentiality
Samples will be stored at -80oC until analysis is complete, after which they will be discarded and destroyed. All data obtained will be dealt with in a confidential manner in line with the Data Protection Act. Data will be held both electronically and in hard copy form for no more than two years before being deleted/destroyed.

I look forward to seeing you, if there are any problems please do not hesitate to call me on one of the numbers below:

Investigator: Amy Tanner
Email:
A.V.Tanner@gre.ac.uk

Supervisor: Dr Judith Allgrove
Email: J.E.Allgrove@gre.ac.uk

Appendix E – LC-MS testosterone exercise samples

Sample	MS
1D Pre	nd
1D Post	nd
1D 15	nd
1D 30	nd
1D 60	nd
2A Pre	nd
2A Post	nd
2A 15	nd
2A 30	nd
2A 60	nd
2D Pre	nd
2D Post	nd
2D 15	nd
2D 30	nd
2D 60	nd
3B Pre	-108.36
3B Post	-103.33
3B 15	18.64
3B 30	21.59
3B 60	160.24
3C Pre	27.72
3C Post	338.01
3C 15	-4.85
3C 30	87.73
3C 60	1.72
4A Pre	19.93
4A Post	-53.71
4A 15	-10.55
4A 30	-161.78
4A 60	-55.18
4D Pre	nd
4D Post	nd
4D 15	nd
4D 30	nd
4D 60	nd
6A Pre	-28.87
6A 15	nd
6A 30	nd
6A 60	nd
6B Pre	31.44

Sample	MS
6B Post	nd
6B 15	nd
6B 30	nd
6B 60	nd
8A Pre	-68.52
8A Post	155.17
8A 15	nd
8A 30	20.26
12A Pre	nd
12A Post	nd
12A 15	nd
12A 30	nd
12A 60	nd
12D Pre	nd
12D Post	nd
12D 15	nd
12D 30	nd
12D 60	nd
13B pre	452.73
13B post	12.26
13B 15	500.57
13B 30	608.19
13B 60	673.03
13C Pre	nd
13C Post	nd
13C 15	nd
13C 30	nd
13C 60	nd
8D pre	nd
8D post	nd
8D 15	nd
8D 30	nd
8D 60	nd
9A pre	nd
9A 15	652.76
9A 30	221.27
9A 60	363.55
9C pre	84.32
9C 15	219.40
9C60	84.32
10A pre	nd
10A post	nd

Sample	MS
10A 15	nd
10A 30	nd
10A 60	nd