LC-ESI-MS/MS quantification of hair cortisol, cortisone and DHEA in combined oral contraceptive users

DS Dorfling

orcid.org 0000-0001-7319-430X

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Supervisor: Prof E Erasmus

Co-supervisor: Dr G Venter

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degrees Celsius
µg	Microgram
µl	Microliter
µm	Micrometer
^{13}C	Carbon 13
2-HP	2-Hydrazinopyridine
ACTH	Adrenocorticotropin hormone
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionisation
AR	Androgen receptor
AUC	Area under the curve
AVP	Arginine vasopressin
BMI	Body mass index
C18	Octadecylsilane
cAMP	Cyclic adenosine monophosphate
CAR	Cortisol awakening response
CBG	Corticosteroid-binding globulin
cm	Centimeter
COC	Combined oral contraceptive
CRH	Corticotropin-releasing hormone
CV	Coefficient of variation
CVD	Cardiovascular disease
CYP	Cytochrome P450
d	Deuterium
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate

DNA	Deoxyribonucleic acid
DNG	Dienogest
DRSP	Drospirenone
E2V	Estradiol valerate
E4	Estetrol
EE	Ethinyl estradiol
ER	Estrogen receptor
ESI	Electrospray ionisation
eV	Electron volts
FSH	Follicle-stimulating hormone
g	Grams
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
H	Hydrogen
H_2O	Water
HairE	Hair cortisone
HairF	Hair cortisol
HairF/DHEA	Hair cortisol to hair DHEA ratio
HairF/E	Hair cortisol to hair cortisone ratio
HDL	High-density lipoprotein
HMP	Hydrazino-1-methylpyridine
HPA	Hypothalamic-pituitary-adrenal
HQC	High concentration quality control
HREC	Health Research Ethics Committee
HSD	hydroxysteroid dehydrogenase
Hz	Hertz
IA	Immunoassay

IMM	Inner mitochondrial membrane
IS	Internal standard
L	Liter
LC	Liquid chromatography
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LNG	Levonorgestrel
LOD	Limit of detection
LQC	Low concentration quality control
m/z	Mass-to-charge ratio
MC2R	Melanocortin 2 receptor
MeOH	Methanol
MetS	Metabolic syndrome
MF	Matrix factor
mg	Milligram
min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimolar
Mol	Mole
MQC	Medium concentration quality control
MR	Mineralocorticoid receptor
MRM	Multiple reaction monitoring
MS	Mass spectrometry
ms	Milliseconds
MS/MS	Tandem mass spectrometry
$NADP^+$	Nicotinamide adenine dinucleotide phosphate

NADPH	Reduced nicotinamide adenine dinucleotide phosphate
ng	Nanogram
NO	Nitric oxide
NOMAC	Nomegestrol acetate
NWU	North-West University
OH	Hydroxy
OMM	Outer mitochondrial membrane
P450c11AS	Aldosterone synthase
P450c11β	11β-Hydroxylase
P450c17	17α-Hydroxylase/17,20-Lyase
P450c21	Steroid 21-hydroxylase
P450scc	Side-chain cleavage enzyme
PCA	Principal component analysis
PCOS	Polycystic ovary syndrome
pg	Picogram
pH	Potential of hydrogen
PKA	Protein kinase A
PMS	Premenstrual syndrome
ppb	Parts per billion
ppm	Parts per million
PR	Progesterone receptor
psi	Pounds per square inch
PVN	Paraventricular nucleus
QC	Quality control
R^2	Correlation coefficient
rpm	Revolutions per minute
RRHD	Rapid resolution high definition
RSD	Relative standard deviation
S/N	Signal-to-noise ratio

SAM	Sympatho-adrenomedullary
SD	Standard deviation
SHBG	Sex hormone-binding globulin
SIM	Single ion monitoring
SPE	Solid phase extraction
StAR	Steroidogenic acute regulatory protein
Th	T helper cells
TNF	Tumour necrosis factor
ULOQ	Upper limit of quantification
USA	United States of America
V	Volts
v/v	Volume: volume ratio %
VTE	Venous thromboembolism
WHR	Waist-to-hip ratio
α	Alpha
β	Beta
γ	Gamma

ABSTRACT

Combined oral contraceptives (COCs) are used chronically by many women worldwide and in South Africa. Because many COC adverse effects coincide with symptoms of stress-related illnesses, including insulin resistance and hypertension, it is believed that these adverse effects are a result of chronic biological stress from long-term COC use and that COC users are at greater risk of developing stress-related illnesses. Different combinations of estrogens and progestins in COCs have varying effects on the stress hormone, cortisol, and stress-related symptoms. Previous studies often do not differentiate between COC compositions and most lack appropriate long-term measurement of biologically active free cortisol. In this study hair cortisol, as a measure of long-term free cortisol, was compared between users and non-users of the commonly used COC consisting of ethinyl estradiol (EE) and drospirenone (DRSP) to determine if COC use results in measurable chronic stress. To get a comprehensive reflection of total cortisol exposure, cortisone, the inactive form of cortisol was quantified as well. Dehydroepiandrosterone (DHEA), a hormone that has anti-cortisol activity, was also intended to be added to reflect total cortisol exposure. An in-house LC-ESI-MS/MS method was developed to simultaneously quantify cortisol, cortisone, and DHEA in hair. The use of ammonium fluoride as a mobile phase modifier enhanced the sensitivity of all analytes without derivatisation. This was, however, insufficient for the low levels of DHEA in hair. Derivatisation was then used to improve DHEA sensitivity but was unfavourable for cortisol. Since cortisol was the most important metabolite in this study, the non-derivatised method was used and DHEA had to be excluded. There were no statistical differences in hair cortisol, cortisone, total glucocorticoids, or the cortisol/cortisone ratio between COC users and non-user controls. These findings suggest that long-term use of COCs containing EE and DRSP does not result in chronic stress or increase the body's exposure to cortisol through altered cortisol synthesis or overall cortisol activity. Further research is needed to understand the mechanisms behind stress-related COC adverse effects. As a secondary aim ethnicity was investigated as a covariate for chronic stress markers in hair. Only the hair cortisol/cortisone ratio differed between Black Africans and Caucasians (p = 0.05, Cohen's d = 1.08), indicating that it should be adjusted for in future studies, particularly in the South African population.

key terms: Combined oral contraceptives, ethinyl estradiol, drospirenone, stress, chronic stress, cortisol, cortisone, DHEA, hair, LC-MS/MS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

CHAPTER 1 INTRODUCTION

1.1 Background

Numerous stressors affect the human body every day. Any threat to normal homeostasis is considered stress. Activation of the hypothalamic-pituitary-adrenal (HPA) axis allows for adaptation to stressors through the action of the hormone cortisol (McEwen, 1998; Nicolaides *et al.*, 2015). Excessive stress experienced over long periods results in chronically elevated cortisol levels, which could be detrimental to health and may lead to several stress-related chronic illnesses, such as type 2 diabetes and cardiovascular disease (Nicolaides *et al.*, 2015; Russell & Lightman, 2019), which greatly contribute to morbidity and mortality worldwide and in South Africa (Jagannathan *et al.*, 2019; Van Zyl *et al.*, 2012). Intake of xenobiotics (foreign chemicals that are exogenously introduced into the body) can disrupt homeostasis and induce a stress response (Lete *et al.*, 2015; Venter *et al.*, 2021). Combined oral contraceptives (COCs) are an example of an exogenous substance frequently used amongst women in the South African population (Boadu, 2022; Yeh *et al.*, 2022). Considering the long-term use of COCs and associated adverse effects, such as hypertension and insulin resistance, it is proposed that the use thereof may result in chronic stress (Eick *et al.*, 2021; Venter *et al.*, 2021). Previous studies investigating the effects of COC use on cortisol levels are inconclusive and lack appropriate long-term stress measures (De Leo *et al.*, 2007; Klipping *et al.*, 2021).

Since it is prolonged cortisol exposure that leads to stress-related illness, an indication of COC's impact on chronic cortisol exposure is necessary. Cortisol is traditionally measured in biological matrices such as serum and saliva, which poses several problems as a chronic stress marker. Serum concentrations of cortisol do not represent the free bioactive levels, although this is not the case for salivary concentrations. Nevertheless, measurements in both of these matrices are only indicative of acute changes in hormone levels and are subject to daily fluctuations from diurnal rhythms and acute stressors. The concentration of cortisol in hair eliminates these issues, as it represents free circulating levels without the influence of daily fluctuations (Russell *et al.*, 2012; Stalder & Kirschbaum, 2012), and would thus give a more reliable reflection of chronic stress in COC users.

The components of COCs, namely, synthetic estrogens and progestins, can also influence cortisol exposure through mechanisms other than stress. These include changes in: cortisol transportation to tissues; conversion to its inactive form, cortisone, within tissues; and the levels of the cortisol antagonist, dehydroepiandrosterone (DHEA) (Klipping *et al.*, 2021; Louw-du Toit *et al.*, 2017; Mattsson & Olsson, 2007; Wiegratz *et al.*, 2003a). Therefore, in combination with hair concentrations of cortisol, levels of cortisone and DHEA could provide a comprehensive

reflection of chronic cortisol exposure in women using COCs. This might aid in gaining clarity on how COC adverse effects arise and help identify women at greater risk for developing stress-related illnesses.

1.2 Problem statement

Numerous studies have indicated that long-term use of COCs is accompanied by several adverse health effects (Lete *et al.*, 2015; Venter *et al.*, 2021). Some, such as hypertension and insulin resistance, coincide with chronic stress symptoms and stress-related illnesses (Nicolaides *et al.*, 2015), suggesting that chronic stress may play a role in adverse side effects and that COC users may be at greater risk of developing stress-related illnesses. Several studies have indicated elevated cortisol levels in COC users (Eick *et al.*, 2021; Klipping *et al.*, 2021). However, findings are contradictory (Ahmed *et al.*, 2011), and disparities may be attributed to the use of an inappropriate chronic stress marker, such as cortisol in serum (Russell et al., 2012). Measurement of hair cortisol concentration overcomes problems with traditional matrices (Wosu *et al.*, 2013), and can allow for improved characterisation of the link between COC use and chronic stress. Moreover, additional markers such as hair cortisone and hair DHEA would give a more comprehensive reflection of total cortisol exposure (Qiao *et al.*, 2017; Zhang *et al.*, 2017). Assessment of hair cortisol, cortisone, and DHEA is lacking in the investigation of the relationship between COC use and chronic stress. Evaluating these hormones in the hair of COC users could be of great value in identifying women at risk of developing stress-related illnesses. Furthermore, ethnicity is likely to have confounding effects on cortisol, cortisone, and DHEA levels in hair, but has not been adequately investigated. Elucidating the relationship between ethnicity and stress markers in hair could help researchers account for covariates in future studies on chronic stress (Wosu *et al.*, 2013).

1.3 Aims and objectives

1.3.1 Aims

The primary aim of this study is to determine if combined oral contraceptive use does indeed result in chronic biological stress. The secondary aim is to assess whether or not ethnicity has a confounding effect on hair markers of chronic stress.

1.3.2 Objectives and Experimental Strategy

The experimental strategy as illustrated in Figure 1-1 includes three objectives that were pursued to reach the aims of this study. The first objective was to develop a selective and sensitive liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) method for the simultaneous quantification of cortisol, cortisone, and DHEA in hair. This was

achieved by optimisation of mass spectrometry and liquid chromatography parameters, as well as the sample preparation procedure. As the second objective, the developed method was validated to ensure its validity for the intended application. Validation included the assessment of selectivity, sensitivity, linearity, accuracy, precision, and stability. The third objective was the implementation of the method on hair samples from Caucasian COC users (n=24), Caucasian COC non-users (n=24), and Black African COC non-users (n=20) to assess their chronic stress profiles. Chronic stress profiles of COC users were compared to those of non-users to determine if COC use results in chronic stress. The chronic stress profiles of Black African non-users were compared to Caucasian non-users to determine if ethnicity has a confounding effect on chronic stress biomarkers in hair.

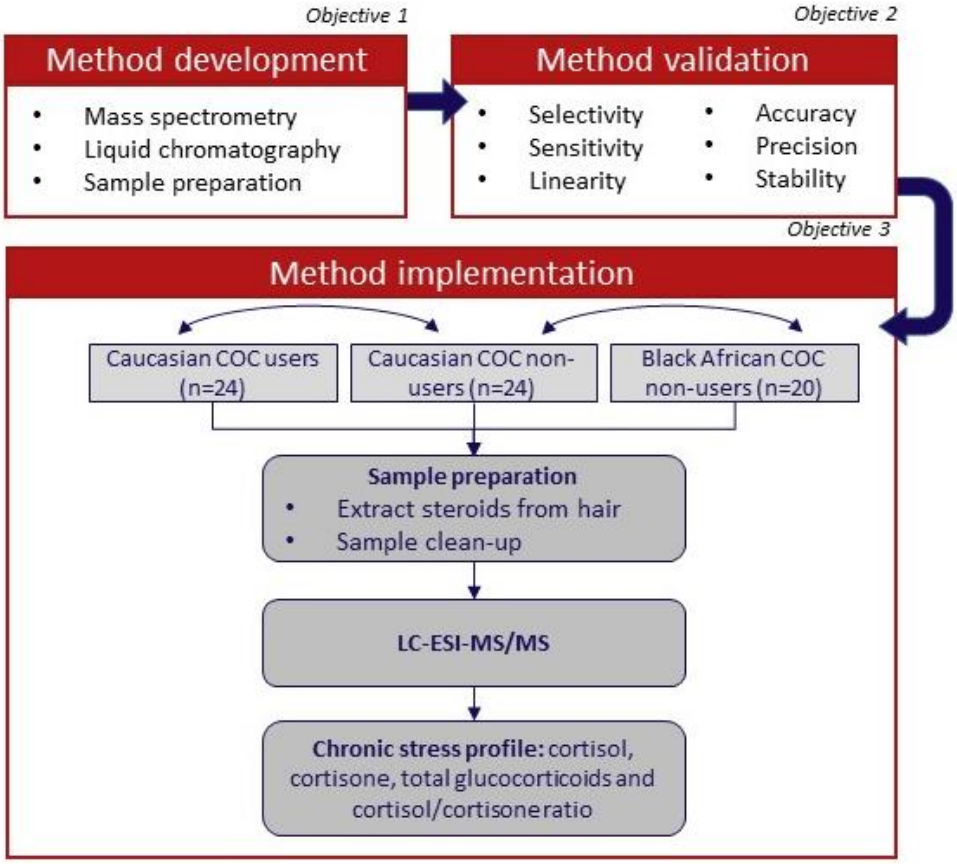


Figure 1-1: Schematic illustration of the experimental strategy indicating the study objectives.

1.4 Dissertation outline

Chapter 2 of this dissertation contains a review of the literature regarding stress hormones, chronic stress, and how oral contraceptive use may lead to chronic stress. The choice of stress

markers in hair and method considerations for their quantification are also discussed in this chapter. Chapter 3 describes the materials and methods used in this study, including those for method development, validation, and implementation. Chapter 4 describes the development and optimisation of the LC-MS/MS method. Chapter 5 describes the validation of the developed method. Chapter 6 deals with application of the method and statistical analysis to compare the different study groups. The conclusion in Chapter 7 highlights the findings of the study, deals with its strengths and weaknesses, and provides future prospects to expand on the knowledge gained.

CHAPTER 2 LITERATURE REVIEW

2.1 Steroids and steroidogenesis

Steroids are lipophilic hormones that originate from cholesterol and have a basic 4-ring structure, three 6-membered rings, and one 5-membered ring (Wudy *et al.*, 2018). Steroids are grouped into classes based on structure and function. Progestogens include pregnenolone and progesterone, which regulates ovulation and maintains pregnancy. Corticosteroids are produced in the adrenal glands and include glucocorticoids and the mineralocorticoid aldosterone. Aldosterone has a role in the control of blood pressure and fluid homeostasis. Glucocorticoids, including cortisol and corticosterone, play a role in metabolism and the stress response. Whereas corticosterone is the main glucocorticoid in mice and several other animals, cortisol is the primary glucocorticoid in humans. Cortisol is central to this study, and as such, its functions will be discussed in more detail further on. Androgens include testosterone and dihydrotestosterone as well as their precursors, androstenedione and dehydroepiandrosterone (DHEA). Estrogens are comprised of estrone, estriol, and estradiol. Testosterone and estradiol are the main sex hormones that have reproductive functions in men and women, respectively (Cole *et al.*, 2019; Louw-du Toit *et al.*, 2016).

2.1.1 Steroidogenesis

The synthesis of steroid hormones, referred to as steroidogenesis (illustrated in Figure 2-1), will now be discussed focusing on those of particular importance in this study, namely DHEA, the glucocorticoids cortisol and cortisone. The adrenal gland, gonads, and placenta are the principal sites of steroid synthesis. However, some other human tissues, including the brain and skin have been found to express steroidogenic enzymes and are capable of synthesising small amounts of steroids (Taves *et al.*, 2011; Thiboutot *et al.*, 2003) The enzymes required for steroidogenesis are various cytochrome P450s (CYPs) and hydroxysteroid dehydrogenases (HSDs) (Miller & Auchus, 2011).

Although a small portion of cholesterol utilised in steroidogenesis is synthesised from acetate, steroids are predominantly synthesised from cholesterol obtained through diet and transported bound to lipoproteins. Cholesterol bound to low-density lipoproteins (LDL) is imported into the cell through receptor-mediated endocytosis. Once released by LDL degradation in the lysosome, free cholesterol is either stored or proceeds to the mitochondria where it can enter the steroidogenic pathway (Miller & Auchus, 2011; Papadopoulos & Miller, 2012). Cholesterol is transported from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM), facilitated by the hormone-responsive steroidogenic acute regulatory protein (StAR) in all steroidogenic cells, except the placenta. At the IMM, conversion of cholesterol to

pregnenolone by hormone-regulated cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) (CYP11A1) is the rate-limiting first step of steroid synthesis (Papadopoulos & Miller, 2012; Prough *et al.*, 2016). Pregnenolone enters the various steroid synthesis pathways through conversion to progesterone by 3β-HSD in the mitochondria or conversion to 17OH-pregnenolone through hydroxylation by 17α-Hydroxylase/17,20-Lyase (P450c17) (CYP17A1) in the endoplasmic reticulum. Progesterone can be utilised in mineralocorticoid or glucocorticoid synthesis, while 17OH-pregnenolone can enter glucocorticoid or androgen synthesis pathways (Miller & Auchus, 2011). Progesterone is converted to aldosterone in the mineralocorticoid pathway, by P450c21 and aldosterone synthase (P450c11AS) (CYP11B2) with 11-deoxycorticosterone, corticosterone, and 18-hydroxycorticosterone as intermediates. In the glucocorticoid pathway, on the other hand, 17-hydroxylation of progesterone by P450c17 or conversion of 17OH-pregnenolone by 3β-HSD yields 17OH-progesterone. Steroid 21-hydroxylase (P450c21) (CYP21A2) then converts 17OH-progesterone to 11-deoxycortisol, which is finally converted by steroid 11β-hydroxylase (P450c11β) (CYP11B1) to cortisol. As further discussed in 2.2.2, cortisol can be converted by 11β-Hydroxysteroid dehydrogenase 2 (11β-HSD2) to cortisone, which can be reconverted to cortisol by 11β-HSD1. 17OH-pregnenolone is alternatively converted to DHEA by the 17,20-lyase activity of P450c17 (Maninger *et al.*, 2009; Miller & Auchus, 2011; Prough *et al.*, 2016). In a series of reactions, DHEA can be converted to other androgens and estrogens (Savineau *et al.*, 2013).

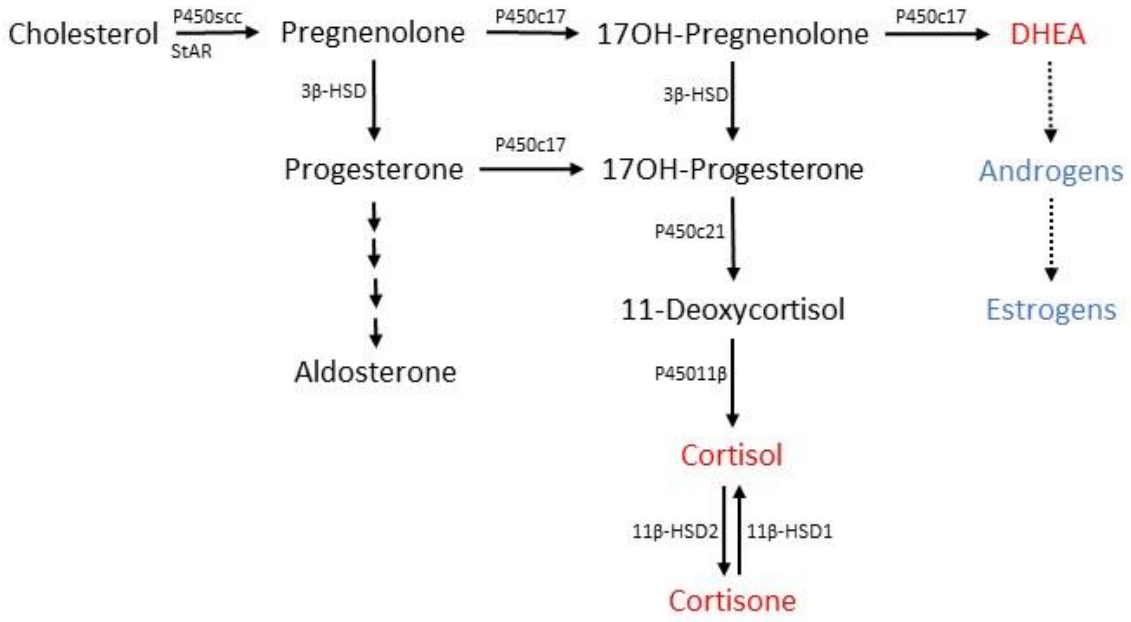


Figure 2-1: Schematic diagram of the steroidogenesis pathway focusing on the synthesis of cortisol, cortisone and DHEA, which are highlighted in red text. Chemical reactions are indicated by arrows, and the enzymes are also shown. Multiple arrows represent the series of reactions and intermediates between progesterone and aldosterone. Blue text represents steroid groups rather than individual steroids, and dotted arrows indicate a range of reactions. Adapted from (Miller & Auchus, 2011).

Many of the enzymes responsible for steroidogenesis catalyse several different reactions. Steroidogenesis occurs in a tissue-specific manner, determined by the activation of different receptors and the distribution of relevant steroidogenic enzymes and their respective cofactors in the different cell types. The adrenal gland contains several cell types that make up distinct zones, which synthesise their primary steroids and minimal amounts of others. Cortisol is predominantly synthesised in the zona fasciculata of the adrenal cortex and aldosterone in the zona glomerulosa, while 80% of DHEA and a small portion of testosterone are produced in the zona reticularis. Estrogens and the remainder of the androgens are synthesised in the gonads, the ovaries and testes respectively (Miller & Auchus, 2011).

2.2 The HPA axis and hormone bioavailability

2.2.1 The HPA axis

Due to the lipophilic nature of steroids, cortisol cannot be stored and thus needs to be rapidly synthesised *de novo* before secretion (Lightman *et al.*, 2020). Synthesis and release of glucocorticoids and DHEA are under the control of the hypothalamic-pituitary-adrenal (HPA) axis, consisting of the hypothalamus, anterior pituitary gland, and the adrenal glands. The paraventricular nucleus (PVN) in the hypothalamus is activated by the circadian clock or stress, inducing secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), which act on corticotroph cells of the anterior pituitary, stimulating the release of adrenocorticotropin hormone (ACTH) (Handa & Weiser, 2014; Kamin & Kertes, 2017; Spencer & Deak, 2017). The binding of ACTH to melanocortin 2 receptor (MC2R) in the adrenals initiates the synthesis and release of cortisol and DHEA (Kamin & Kertes, 2017; Spencer & Deak, 2017). The action of ACTH in the zona fasciculata controls cortisol synthesis by an acute response through activation of StAR, as well as a chronic response that increases the expression of steroidogenic genes. Upon ACTH binding, MC2R activates the adenylyl cyclase cascade leading to phosphorylation of StAR by protein kinase A (PKA), thereby activating it for cholesterol import to the IMM. PKA also phosphorylates and activates the transcription factors responsible for gene expression of steroidogenic enzymes and other proteins, including StAR, CYP11A1, and MC2R (Gallo-Payet *et al.*, 2017; Lightman *et al.*, 2020). Negative feedback of glucocorticoid secretion occurs at the HPA level as well as at the adrenal level. High glucocorticoid levels suppress CRH, ACTH, and cortisol by activation of glucocorticoid receptors (GR) in the hypothalamus, pituitary, and adrenals, respectively. Negative feedback serves to protect against the harmful effects of excess glucocorticoids which will be further discussed later on (Nicolaides *et al.*, 2017; Walker *et al.*, 2015). In contrast, DHEA does not exert negative feedback on the HPA axis (Kamin & Kertes, 2017).

Under basal conditions, cortisol is secreted in a daily oscillating pattern. Just before waking, cortisol levels start rising and suddenly increase to the highest levels 30-45 min after waking. This sudden increase is known as the cortisol awakening response (CAR). Thereafter, levels decline throughout the day with the lowest level (nadir) at night (Russell & Lightman, 2019; Stalder *et al.*, 2016). This pattern is known as the circadian rhythm. It is an important component linking metabolism and the stress response to the circadian clock system, the purpose of which is to adjust homeostatic control in accordance with activities and requirements throughout the day/night cycle. In the hypothalamus, the suprachiasmatic nucleus of the circadian clock receives light input throughout the day and activates the PVN for CRH release. Furthermore, StAR gene transcription is also regulated by the circadian rhythm, causing a circadian pattern of StAR expression and adrenal sensitivity (Lightman *et al.*, 2020; Nicolaides *et al.*, 2017). DHEA exhibits a similar, albeit less pronounced, diurnal pattern, but lacks the spike of the CAR (Hucklebridge *et al.*, 2005). Another pattern that is evident in cortisol secretion, is the ultradian rhythm characterised by a pulse of glucocorticoid secretion every 60-90 min. Regulation of the circadian and ultradian rhythms are independent of one another and the ultradian rhythm is not regulated at the PVN level (Waite *et al.*, 2012). Instead, the pulsatile pattern seems to be regulated by oscillating ACTH due to a balance between feedforward stimulation by CRH, and momentarily delayed negative feedback from glucocorticoids acting on the pituitary (Walker *et al.*, 2012). The ultradian rhythm maintains HPA responsiveness, since downregulation of steroidogenic gene transcription would result from continuous activation of the system (Hazell *et al.*, 2019; Lightman *et al.*, 2020).

2.2.2 Cortisol and DHEA(S) Bioavailability

Levels of circulating hormones that are biologically active are regulated by several factors, including synthesis, transportation, conversion between active and inactive forms, and excretion from the body. This is true for both cortisol and DHEA.

Once synthesised and secreted under control of the HPA axis, cortisol must be transported to target tissues. Cortisol is transported in the circulation as free cortisol (5-6%) or bound to the carrier proteins corticosteroid-binding globulin (CBG) and albumin (80-90% and 10-15%, respectively). Only free cortisol is believed to be biologically active and CBG has an important role in regulating free cortisol levels in the blood (Bae & Kratzsch, 2015). Circulating levels of bioactive cortisol can be altered through changes in CBG concentration and cortisol binding properties. Decreased CBG production in the liver or a lower affinity for cortisol binding will increase the fraction of free cortisol in the plasma (Bae & Kratzsch, 2015; Henley *et al.*, 2016; Meyer *et al.*, 2016). There are two forms of CBG in the circulation – a portion of the high-affinity CBG produced in the liver is cleaved by neutrophil elastase into low-affinity CBG (Nenke *et al.*, 2017). The affinity of CBG for cortisol can also be altered by changes in temperature and pH,

possibly playing a role in targeted delivery at inflammation sites (Breuner *et al.*, 2020; Meyer *et al.*, 2016).

Interconversion between cortisol and its inactive form, cortisone, occurs in some tissues (Miller & Auchus, 2011). 11β-HSD2 oxidises active cortisol to inactive cortisone in mineralocorticoid-responsive tissues, protecting mineralocorticoid receptors from excessive nonspecific glucocorticoid activation (Zhang *et al.*, 2013). 11β-HSD1 can catalyse the reaction in both directions depending on the availability of $NADP^+$ or NADPH as cofactors. However, *in vivo*, the reduction of cortisone to cortisol is favoured (Tomlinson & Stewart, 2001). 11β-HSD1 is expressed in various tissues, including adipose tissue, gonads, liver, and some brain regions (cerebellum and hippocampus) where it amplifies levels of active cortisol to enhance glucocorticoid sensitivity (Cooper & Stewart, 2009; Miller & Auchus, 2011).

DHEA is most abundant in the circulation in its sulphated form, dehydroepiandrosterone sulphate (DHEAS). Both forms are biologically active and coexist in the circulation. Sulfonation is catalysed by sulfotransferase, while steroid sulfatase catalyses the reverse reaction (Kamin & Kertes, 2017). DHEA and DHEAS are transported in the circulation mostly bound to albumin, while a small portion is bound to sex hormone-binding globulin (SHBG), leaving approximately 5% as free and biologically active. DHEA and DHEAS will further be referred to collectively as DHEA(S) unless otherwise stated.

2.3 Cortisol and DHEA Functions

2.3.1 Cortisol functions

Glucocorticoids exert their wide range of tissue-specific functions via both genomic and non-genomic mechanisms. Genomic mechanisms act through the binding of glucocorticoids to nuclear GRs and mineralocorticoid receptors (MRs). The receptor is then imported into the nucleus, where it interacts with deoxyribonucleic acid (DNA) to induce or inhibit the expression of target genes. The GR acts as a transcription factor that binds to glucocorticoid response elements (GREs) in DNA, initiating transcription of target genes. Another way that GRs regulate gene expression is through interaction with other transcription factors, either inhibiting or enhancing their activity (Cain & Cidlowski, 2017; Kamin & Kertes, 2017; Nicolaides *et al.*, 2015). Cell-specific functions are due to the recruitment of different transcriptional machinery and interaction with different GRE sequences (Kuo *et al.*, 2015; Nicolaides *et al.*, 2015). Non-genomic actions of glucocorticoids act through kinase signalling cascades upon binding to GRs and MRs located in the cell membrane. These actions occur within seconds to minutes, compared to genomic actions that may take several hours (Nicolaides *et al.*, 2015). MR has up to 10 times greater affinity for cortisol than GR does. Furthermore, membrane-associated MR

and GR have lower glucocorticoid affinities than cytoplasmic GR and MR. This means that at low cortisol levels, nuclear MR is mostly activated whereas higher glucocorticoid levels are required for activation of nuclear GR, and membrane-bound GR and MR, such as at ultradian and circadian peaks and during stress (Lightman *et al.*, 2020; Russell & Lightman, 2019). Whereas GR is expressed in most tissue types, expression of MR is restricted to only a few tissues including adipose tissue, kidneys, and several brain regions (Cain & Cidlowski, 2017; Lightman *et al.*, 2020).

Glucocorticoids are important in maintaining homeostasis and are a significant component of the body's stress response. They play pivotal roles in the metabolic, nervous, cardiovascular, and immune systems. The metabolic functions of cortisol serve to elevate circulating glucose levels, an important requirement during stress and fasting. Firstly, this is achieved by inhibiting insulin production in the pancreas and decreasing glucose uptake and utilisation in skeletal muscle and adipose tissue. Secondly, cortisol stimulates gluconeogenesis in the liver, increasing glucose production from non-carbohydrate substrates such as glycerol, lactate, fatty acids, and gluconeogenic amino acids. Glycerol, fatty acids, and amino acids for gluconeogenesis are supplied by cortisol-induced lipolysis, decreased fatty acid oxidation, and skeletal muscle degeneration (Kuo *et al.*, 2015; Vegiopoulos & Herzig, 2007). Glucocorticoids have effects on the nervous system through modulation of glutamate, dopamine, serotonin, and noradrenaline neurotransmission in several brain regions, particularly in the hippocampus (Joca *et al.*, 2007). During stress, fast nongenomic MR actions in certain brain regions lead to improved cognition and decision-making (Russell & Lightman, 2019). Both GR and MR are expressed in the prefrontal cortex, amygdala, and hippocampus, among other brain regions. These are regions involved in learning and memory, where MR activity is involved in memory retrieval and GR activation leads to memory consolidation. Memory retrieval is however impaired by GR activation during stress (Lightman *et al.*, 2020). Cortisol controls blood pressure by enhancing the production of vasoconstrictors such as endothelin-1 and acetylcholine, while concomitantly decreasing the release of vasodilators such as nitric oxide (NO). Cortisol enhances platelet production, coagulation, and fibrinolysis to prevent excessive bleeding if injured (Sandrini *et al.*, 2020). Low levels of glucocorticoids have immune-promoting actions, but higher glucocorticoid concentrations have immunosuppressive effects. Cain and Cidlowski (2017) propose that this maintains the sensitivity of the immune system so that the immune and inflammatory response can be induced when infection or damage occurs, but once it has been initiated, glucocorticoids shorten the duration of the response. Cortisol suppresses adaptive immunity, T helper (Th) 1 cell function, and antibody production while enhancing innate immunity and Th2 cell activity (Cain & Cidlowski, 2017).

2.3.2 Anti-glucocorticoid action of DHEA(S)

DHEA(S) has both genomic and non-genomic actions, but distinct receptors for DHEA(S) have not yet been discovered. Although DHEA does bind to estrogen receptors (ERs) and androgen receptors (ARs), several genomic actions have been shown to be independent of their activation (Savineau *et al.*, 2013). Non-genomic actions occur through regulation of voltage-gated channels, alterations in plasma membrane properties, and neurotransmitter release (Kamin & Kertes, 2017).

DHEA(S) was first thought to be only an intermediate of testosterone and estrogen synthesis, but is now known to have physiological actions that are independent of its conversion to sex hormones. The functions of DHEA(S) have not been fully elucidated, but it has several known actions that largely oppose those of glucocorticoids (Kamin & Kertes, 2017; Maninger *et al.*, 2009). DHEA stimulates glucose uptake, glycolysis, and fatty acid oxidation in skeletal muscle cells (Sato *et al.*, 2008). In the nervous system, DHEA(S) promotes neuronal generation and survival. Although DHEA inhibits glutamate transmission, it acts as a neurotransmitter that stimulates glutamate receptors but antagonizes receptors for the inhibitory neurotransmitter γ-Aminobutyric acid (GABA) (Kamin & Kertes, 2017; Maninger *et al.*, 2009). In the cardiovascular system, DHEA stimulates the release of NO from endothelial cells, having a vasodilatory effect (Sandrini *et al.*, 2020). Furthermore, DHEA has antioxidant effects (Savineau *et al.*, 2013). DHEA(S) is anti-inflammatory and strengthens the immune response to pathogens. It inhibits proinflammatory cytokine production, T-cell and natural killer cell cytotoxicity, and promotes apoptosis (Prall & Muehlenbein, 2018; Savineau *et al.*, 2013). The mechanisms through which DHEA exerts its anti-glucocorticoid actions are unclear. It is not due to interference with cortisol binding GR and MR, but can be partly explained by upregulation of 11β-HSD 2 with concomitant downregulation of 11β-HSD1 (Balazs *et al.*, 2008), as well as competition between 7α-hydroxy-DHEA and cortisone for 11β-HSD1, leading to decreased conversion of cortisone to active cortisol (Muller *et al.*, 2006).

2.4 The stress response

Aside from the daily cortisol secretion patterns, additional cortisol is secreted during stress. To understand cortisol's role in stress we first need to understand what stress is. Stress is any stimulus that causes the disruption of homeostatic control or that is perceived as a threat to homeostasis. Stress can be physiological or psychological (Russell & Lightman, 2019). When stress is experienced, the stress system is activated in an attempt to restore homeostasis through several physiological changes. This process is referred to as allostasis (McEwen, 1998). The stress system is composed of the sympatho-adrenomedullary (SAM) axis and HPA axis. During the stress response, behavioural changes occur quickly, then SAM is activated

within seconds to release epinephrine and norepinephrine, followed by HPA-activated cortisol and DHEA release after 15-20 minutes (Kamin & Kertes, 2017; Nicolaides *et al.*, 2015; Russell & Lightman, 2019; Tsigos & Chrousos, 2002). Once the stress has passed, the stress system is no longer activated and negative feedback allows cortisol to subside to normal levels (Russell & Lightman, 2019).

The roles that glucocorticoids play in the stress response are manifold. Briefly, cortisol brings about behavioural changes such as heightened alertness and enhanced cognitive thinking. It induces a metabolic shift from anabolism to catabolism, which together with increased heart rate and blood pressure, provides extra energy and oxygen to the brain, muscles, and stress sites. Moreover, inhibition of non-crucial functions such as feeding, reproduction, and growth conserves energy for more essential processes (Iob & Steptoe, 2019; Kamin & Kertes, 2017; Nicolaides *et al.*, 2015; Russell & Lightman, 2019; Tsigos & Chrousos, 2002). The role that DHEA plays in stress mediation has not been fully elucidated but it appears to restrain excess glucocorticoid activity which could have negative health impacts (Kamin & Kertes, 2017).

2.5 Cortisol dysregulation and chronic stress

It can be seen from the above that glucocorticoids are crucial to homeostatic maintenance, but like many other physiological systems, dysregulation of the HPA axis may be detrimental. Cortisol homeostasis can be altered by endocrine disorders as well as chronic stress.

Cushing's syndrome is characterised by hypercortisolism as a result of excessive ACTH secretion or ACTH-independent adrenal secretion of cortisol, usually due to pituitary or adrenocortical adenomas, respectively (Raff & Carroll, 2015). In Addison's disease, insufficient cortisol is released due to impaired cortisol synthesis or adrenal damage (Ten *et al.*, 2001). Cortisol dysregulation is not only in the form of increased or decreased cortisol levels. Disruption of pulsatile and circadian secretion patterns as seen in obstructive sleep apnoea and during the use of synthetic glucocorticoids leads to interference of daily cortisol activities (Lightman *et al.*, 2020).

The stress system becomes maladaptive if the stress response is excessive or is not shut off after the stressful event has passed, or when stress is experienced repetitively or for a prolonged time (Nicolaides *et al.*, 2015). During chronic stress, negative feedback on the HPA axis is attenuated by downregulation of GR in relevant brain regions, further elevating cortisol secretion (Kamin & Kertes, 2017; Russell & Lightman, 2019). Moreover, increased synthesis of cortisol during chronic stress redirects steroidogenesis away from DHEA production, resulting in reduced DHEA(S) levels (Kamin & Kertes, 2017). Prolonged exposure to elevated glucocorticoid levels no longer aids in stress adaptation, but rather gives rise to damaging

effects on the body. This is referred to as allostatic load (McEwen, 1998). Chronic stress contributes to the development of a variety of stress-related illnesses such as metabolic syndrome (MetS), type 2 diabetes mellitus, cardiovascular disease (CVD), hypertension, immune deficiency, depression, and some types of cancer. MetS is characterised by obesity, insulin resistance, hypertension, hyperglycaemia, dyslipidaemia, and muscle atrophy. Insulin resistance due to interrupted insulin signalling by tumour necrosis factor (TNF) activation further increases the risk for type 2 diabetes mellitus. Dyslipidaemia presents as high-density lipoprotein (HDL)-cholesterol with increased LDL-cholesterol, total cholesterol, and triglycerides. Increased lipolysis of peripheral fat and hypertrophy of visceral fat lead to visceral obesity, which further contributes to insulin resistance (Kuo *et al.*, 2015; Vegiopoulos & Herzig, 2007). Cortisol-induced decrease in growth hormone leads to loss of bone and muscle mass. Increased gluconeogenesis contributes to hyperglycaemia and aggravates muscle wasting (Nicolaides *et al.*, 2015; Vegiopoulos & Herzig, 2007). The prolonged cortisol-induced switch from Th1 to Th2 puts chronically stressed individuals more at risk of infections and autoimmune disorders (Nicolaides *et al.*, 2015), while diminished lymphocyte and antibody production slows down illness recovery (Kamin & Kertes, 2017). In addition to the contributions of dyslipidemia, hyperglycemia, and insulin resistance, hypertension is caused by sodium retention, expansion of plasma volume, and vascular inflammation (Iob & Steptoe, 2019; Marvar *et al.*, 2012). Increased vasoconstriction with decreased vasodilation for prolonged periods results in endothelial dysfunction. Chronic stress is associated with thrombosis due to enhanced coagulation and diminished fibrinolysis (Sandrini *et al.*, 2020). Reduced dopamine levels and disrupted serotonergic transmission in the hippocampus, together with hippocampal neuron death due to excess glutamate release, contribute to the development of depression (Joca *et al.*, 2007).

The chronic stress-related decline of DHEA production may be important in the aetiology of stress-related disorders, through insufficient glucocorticoid-resisting activity (Kamin & Kertes, 2017). Low DHEA(S) levels have been related to CVD risk (Savineau *et al.*, 2013), and the cortisol/DHEAS ratio is raised in MetS (Phillips *et al.*, 2010) type 2 diabetes (Fernández *et al.*, 2020) hypertension (Carroll *et al.*, 2011), and depression. Elevated cortisol/DHEA ratio is also associated with increased risk of infection after injury (Kamin & Kertes, 2017).

2.6 Hair cortisol, cortisone, and DHEA as markers for chronic stress

Cortisol is a biomarker used for the diagnosis of HPA abnormalities, such as Cushing's syndrome and Addison's disease. Because of its prominent role in stress mediation, it is also an excellent stress biomarker, which may be used in the prognosis of stress-related illnesses. Cortisol monitoring can aid in early intervention for abnormalities, and possibly prevention of stress-related illnesses. Cortisol is quantified in various biological matrices, with different

applications and limitations. (Greff *et al.*, 2019). Traditionally, quantification is performed in serum and saliva. Since the majority of cortisol transported in the blood is bound to carrier proteins, most measurements in serum are reflective of total cortisol and not biologically active free cortisol (Russell *et al.*, 2012). To calculate free cortisol, it must be separated from CBG-bound cortisol, which is time-consuming and different methods do not yield equivalent results (Pretorius *et al.*, 2011). Moreover, venepuncture involved in serum sampling is invasive and may cause a stress-induced spike in cortisol levels (Weckesser *et al.*, 2014). On the other hand, salivary cortisol offers the ability to quantify free cortisol, due to passive diffusion of cortisol into saliva. Sample collection is less invasive than that of serum and samples may be collected at home by the participants themselves. However, problems may arise with compliance to sampling procedures in regard to timing (Kudielka *et al.*, 2012; Stoffel *et al.*, 2021). Measurements in these matrices are often taken at single time points, for which sample collection is time-sensitive, and cortisol fluctuations throughout the day may lead to inaccuracies. To overcome this, samples are taken at the same time of day, or certain features of the circadian pattern, such as CAR, are selected for comparability (Kudielka *et al.*, 2012; Stalder *et al.*, 2016). Nevertheless, short-term measurements are only indicative of acute changes in cortisol secretion, allowing for analysis of HPA axis responsiveness to stress (Stoffel *et al.*, 2021).

Although acute indices of cortisol are meaningful in some studies, others may require long-term measurements as an indication of basal HPA axis function for monitoring chronic stress. This can be achieved by taking multiple saliva samples at various points throughout the day or collecting 24-hour urinary samples. From these, the average daily cortisol and area under the curve (AUC) can be used to assess total cortisol secretion over a longer time, while also accounting for variations throughout the day (Kudielka *et al.*, 2012; Stoffel *et al.*, 2021). Day-to-day variation remains, and therefore, averages of several days may be necessary for the portrayal of long-term cortisol exposure (Zhang *et al.*, 2017). Not only does this make sampling unnecessarily time-consuming and costly, but also intensifies the stress that participants may experience. For this reason, an alternative marker emerged. Hair cortisol concentration (further referred to as hairF) has become increasingly used as a chronic stress marker since the observation by Kirschbaum *et al.* (2009) that cortisol in hair can display long-term cortisol production. As in saliva, unbound cortisol enters the hair predominantly by passive diffusion from the blood representing biologically active free cortisol (Hobo *et al.*, 2021; Russell *et al.*, 2012). However, a small portion of hair cortisol may be contributed by local synthesis in the hair follicle and by application of exogenous glucocorticoids, but this is negligible. Cortisol is incorporated into the hair over a long time, eliminating the effects of intra-day and inter-day fluctuations that are seen in traditional matrices (Russell *et al.*, 2012). Based on the average 1 cm/month growth rate of hair, which should be adapted for varying growth rates in different

ethnicities (Loussouarn *et al.*, 2016), samples can be divided into sections to retrospectively assess the time period of interest. However, observation is limited to 6 months because of decreased cortisol levels past the proximal 6 cm of the hair shaft, resulting from hair damage and increased cortisol wash-out in more distal sections (Kirschbaum *et al.*, 2009; Stalder & Kirschbaum, 2012). Additional advantages are that sampling is less invasive and samples can be stored for several months at room temperature, which is not the case for the other sample matrices (Greff *et al.*, 2019; Stalder & Kirschbaum, 2012). The correlation of hairF with cortisol in other matrices has been investigated. A study by Zhang *et al.* (2018) found no correlation between short-term cortisol indices in salivary cortisol, but the strength of the association increased with longer-term measurements, such as single-day and three-day averages (Zhang *et al.*, 2018). Furthermore, hairF is also highly consistent with 30-day salivary but not urinary cortisol (Short *et al.*, 2016; Sugaya *et al.*, 2020). However, there is a moderate correlation with urinary cortisol over 2 months (Van Ockenburg *et al.*, 2016). Therefore, hairF is a suitable marker for long-term but not short-term cortisol exposure, and elevated hairF has been associated with various stressors and stress-related illnesses (Greff *et al.*, 2019; Wester & van Rossum, 2015).

Cortisone is occasionally measured as an alternative to cortisol or in combination with it. Although cortisone is not an active glucocorticoid, salivary, urinary, and hair cortisone correlate to their active counterparts in these matrices over short-term and long-term measurements, presenting as an alternative biomarker (Zhang *et al.*, 2017). Salivary cortisone has also been shown to reflect free cortisol in serum (Perogamvros *et al.*, 2010). The presence of very low hairF makes detection challenging, while cortisone is present at concentrations approximately 3-4 times higher than cortisol, allowing it to be more easily quantified (Zhang *et al.*, 2018). Alternatively, the two hormones can be quantified simultaneously to evaluate the activity of the 11β-HSD enzymes and give a more wholistic depiction of the active and inactive glucocorticoids (Zhang *et al.*, 2017). An increased cortisol/cortisone ratio is associated with stress and CVD (Chen *et al.*, 2014). As with the relationship between hair and salivary cortisol, hair cortisone (hairE) levels are comparable to those of long-term salivary cortisone, but are inconsistent with acute measurements, demonstrating the validity of hair cortisone as an indication of chronic stress (Zhang *et al.*, 2018). Moreover, the hairF/E ratio correlates to the salivary ratio (Condon *et al.*, 2019). Again, the retrospective time frame is limited due to hormone washout in more distal sections of the hair shaft (Zhang *et al.*, 2013).

The contrasting actions of cortisol and DHEA(S) merit the use of the cortisol/DHEA(S) ratio as a marker to portray overall glucocorticoid activity (Kamin & Kertes, 2017). Increased cortisol/DHEA ratios in saliva and serum have been associated with stress such as childhood maltreatment and racial discrimination (Lee *et al.*, 2021; Van Voorhees *et al.*, 2014).

Furthermore, increased salivary cortisol/DHEA(S) ratios in saliva were associated with stress in caregivers of Alzheimer's patients compared to controls, when cortisol levels alone were not associated (Jeckel *et al.*, 2010). Like cortisol, measurements in traditional matrices may be influenced by diurnal DHEA secretion patterns, implying that hairDHEA(S) and its relationship with hairF could be relevant in chronic stress assessment. HairDHEA and the hairF/DHEA ratio correlate to the one-day average of salivary levels. HairF/DHEA was elevated in perceived stress and exposure to stressful early life events (Qiao *et al.*, 2017). Increased hairF/DHEA ratio has also been associated with depression (Young *et al.*, 2002).

2.7 Covariates of hair biomarkers

When designing a study, potential covariates for the intended biomarkers need to be considered and accounted for. Several covariates have been identified for hairF, but not all findings are consistent. The first factor to consider is the physical properties of the hair. Natural hair colour has no effect in most studies. Hair washing frequency has no effect in proximal sections, but the previously mentioned washout effect is enhanced in distal sections with more frequent hair washing. Contradictory reports exist for the effect of hair treatment with dyes or bleach, with most reporting no significant association. However, regular heat treatments, such as the use of hair straighteners, are associated with lower hairF. Lifestyle factors such as alcohol consumption and smoking have also been found to not affect hairF levels. For demographic variables, correlations have been found for sex and age, with higher cortisol in men than in women. Higher body mass index (BMI) and waist-to-hip ratio (WHR) are associated with higher hairF and a non-linear relationship exists with age (Fischer *et al.*, 2017; Rippe *et al.*, 2016; Staufenbiel *et al.*, 2015; Wosu *et al.*, 2015; Wosu *et al.*, 2013; Zhang *et al.*, 2013). Only a few studies have investigated the effects of ethnicity. HairF was found to be higher in black and Hispanic ethnic groups than in Caucasian ethnic groups in the United States of America (Wosu *et al.*, 2015). A study conducted by Abell *et al.* (2016) in London had similar findings with higher hairF in black ethnic groups compared to Caucasian and Asian ethnic groups. In both studies, the authors suggested that differences were attributed to stress associated with being in a minority group, rather than biological variation (Abell *et al.*, 2016; Wosu *et al.*, 2015). The contribution of socioeconomic stress and racial discrimination to increased hairF in black subjects in a USA population was later confirmed (Lehrer *et al.*, 2020). Reports of higher blood cortisol in Caucasian South African women compared to black South African women (Schutte & Olckers, 2007; Tolmay *et al.*, 2012) suggests that disparity may exist between hairF in South African women and other reports of ethnicity as a hairF covariate. The factors affecting hairE have not been as extensively evaluated. Age and sex affect hairE similarly to hairF. Darker hair colours are associated with higher hairE and more frequent hair washing is associated with lower hairE, while no association was observed with BMI, waist circumference, smoking, or

alcohol consumption (Musana *et al.*, 2020; Staufenbiel *et al.*, 2015; Wester & van Rossum, 2015), whereas race and ethnicity have not been investigated. Limited research has been done on covariates of hairDHEA. Findings suggest that hairDHEA is not affected by hair colour, hair treatment, hair washing frequency, or BMI (Ilg *et al.*, 2020; Lehrer *et al.*, 2016; Qiao *et al.*, 2017). Seasonal variation, with higher levels in winter than summer, has been observed in hairF and hairE, but is not evident in hairDHEA (Chen *et al.*, 2019).

2.8 Combined oral contraceptives and cortisol

2.8.1 Combined oral contraceptive use and adverse effects

Combined oral contraceptives (COCs) consisting of a combination of synthetic estrogens and progestins are amongst the most commonly used contraceptive methods worldwide and in South Africa (Boadu, 2022; Yeh *et al.*, 2022). COCs regulate endogenous estrogen and progesterone levels and inhibit ovulation through suppression of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Dragoman, 2014). They are primarily used to prevent unwanted pregnancy, but other non-contraceptive uses include treatment of polycystic ovary syndrome (PCOS), acne, hirsutism premenstrual syndrome (PMS), dysmenorrhea, and endometriosis (Dragoman, 2014; Regidor & Schindler, 2017). Common adverse effects associated with COC use are nausea, dizziness headache, weight gain, increased irritability, and breast tenderness (Bachmann & Kopacz, 2009; Dragoman, 2014). COC use is also associated with more serious, albeit less prevalent, conditions such as depression, insulin resistance, venous thromboembolism (VTE), and breast cancer (de Wit *et al.*, 2020; Lete *et al.*, 2015; Rodrigo *et al.*, 2011; Sitruk-Ware & Nath, 2011; Smrekar & Lodise, 2022).

The structure of some progestins allows for binding other steroid receptors besides the progesterone receptor (PR), which partly contributes to COC adverse effects (Sitruk-Ware & Nath, 2011). COCs usually contain estrogens such as ethinyl estradiol (EE) or estradiol valerate (E2V) in combination with progestins, including levonorgestrel (LNG), norethindrone, dienogest (DNG) gestodene, nomegestrol acetate (NOMAC) or drospirenone (DRSP), most of which are structurally related to testosterone or progesterone (Dragoman, 2014; Oelkers, 2004; Sitruk-Ware & Nath, 2011). This study focuses on the use of COCs containing EE and DRSP. DRSP is amongst the newer generation of progestins, that was developed to minimise off-target adverse effects through reduced binding to other steroid receptors (Louw-du Toit *et al.*, 2017). DRSP, derived from the MR antagonist spironolactone, is more similar to progesterone than other progestins, with improved PR affinity, lower AR, and no ER affinity. DRSP has no estrogenic or glucocorticoid activity, but is antiandrogenic (Oelkers, 2004; Regidor & Schindler, 2017). Lower androgenic activity of DRSP improves lipid profiles with lower LDL-cholesterol and higher HDL-cholesterol compared to the androgenic progestin LNG (Lete *et al.*, 2015). Moreover, its anti-

mineralocorticoid activity counteracts the EE-induced aldosterone secretion, causing lower blood pressure and reduced weight gain compared to COCs containing EE paired with LNG or DNG – progestins that do not have anti-mineralocorticoid activity (Oelkers, 2004).

Despite the reported improved safety profile of EE/DRSP compared to formulations with other progestins, the high risk of VTE persists as well as elevated triglycerides and insulin resistance, which are considered risk factors for CVD and diabetes mellitus type 2 (Klipping *et al.*, 2021; Lete *et al.*, 2015). Moreover, our research group found elevated blood pressure, fatigue, and reduced physical health status (more reported medical symptoms) in women using COCs containing EE and DRSP compared to women not using contraceptives. (Venter *et al.*, 2021). The mechanisms underlying COC adverse effects are not fully understood. Oxidative stress observed in COC users has been implicated (De Groote *et al.*, 2009; Venter *et al.*, 2021), since oxidative stress is implicated in the pathology of several disease states, including hypertension and diabetes (Forman & Zhang, 2021). Similarities between adverse effects and symptoms of stress-related illnesses suggest that cortisol has a role in EE/DRSP adverse effects.

2.8.2 COCs and cortisol

Intake of COCs disrupts many homeostatic parameters (Lete *et al.*, 2015; Venter *et al.*, 2021) suggesting that COC use may lead to biological stress. The influence of COCs on not only glucocorticoid levels, but also their activity, may give rise to negative health consequences. Considering the long-term use of COCs, their association with chronic cortisol exposure may aid in elucidating the aetiology of COC adverse effects and identify women at risk of developing stress-related illnesses.

Some studies have found altered lipid metabolism (increased triglycerides and total cholesterol) and increased insulin resistance in combination with elevated cortisol in COC users (Eick *et al.*, 2021; Hertel *et al.*, 2017; Klipping *et al.*, 2021; Macut *et al.*, 2015). Eick *et al.* (2021) and Hertel *et al.* (2017) investigated the association between some of these parameters and found that increased triglycerides, altered phospholipid profile, and decreased amino acids were significantly associated with cortisol levels (Eick *et al.*, 2021). Nevertheless, participants in both studies could be using any COC, so results may not be directly translatable to EE/DRSP-containing COCs, since different COC formulations have been shown to impact cortisol levels to varying degrees (Ågren *et al.*, 2011; Klipping *et al.*, 2021; Wiegratz *et al.*, 2003a). Reports of EE/DRSP effects on serum/plasma cortisol are inconsistent. Some indicated that cortisol levels were increased from baseline after several months of EE/DRSP use (Klipping *et al.*, 2021; Macut *et al.*, 2015), while another indicated that they were unaltered (De Leo *et al.*, 2007). Interestingly, a study by Ahmed *et al.* (2011) found increased plasma cortisol but decreased urinary cortisol after three weeks of EE/DRSP use. Conflicting results might be due to the

choice of biological matrix that does not allow accurate assessment of chronic cortisol exposure because of possible fluctuations at the time of sampling.

Louw-du Toit *et al.* (2017) suggests that different progestins have cortisol-modulating actions through the alteration of steroid synthesis pathways. Some progestins have been shown to alter the synthesis of several other steroids as well. In vivo animal studies (rats and fish) showed varying effects of different progestins (including LNG, norethisterone, and medroxyprogesterone acetate) on levels of pregnenolone, progesterone, and 17OH-progesterone (Louw-du Toit *et al.*, 2017), all of which are cortisol precursors (Miller & Auchus, 2011). Louw-du Toit *et al.* (2016) investigated the effects of multiple progestins on levels of various steroids in the steroidogenic pathway of H295R human adrenocortical carcinoma cells, which express all the enzymes required for steroidogenesis. Treatment with DRSP decreased levels of cortisol and its precursor, 11-deoxycortisol, and increased progesterone and 17OH-progesterone. Upon investigation of enzymatic activity, 3β-HSD2 activity and 17α-hydroxylase activity of CYP17A1 were found to be inhibited by DRSP in COS-1 cells transfected with 3β-HSD2 and CYP17A1 expression vectors. Inhibition of these enzymes explained the downstream decrease in cortisol and 11-deoxycortisol and the upstream elevated levels of progesterone and 17OH-progesterone Louw-du Toit *et al.* (2016). De Leo *et al.* (2007) investigated steroidogenesis in response to ACTH in women with PCOS after 3 months of EE/DRSP use. Although 17OH-progesterone/progesterone and androstenedione/17OH-progesterone ratios were decreased, indicating that both 17α-hydroxylase and 17/20-lyase activities of CYP17A1 were inhibited, serum cortisol levels were unaltered (De Leo *et al.*, 2007).

It is widely known that estrogens increase cortisol levels in a dose-dependent manner (Wiegratz *et al.*, 2003b) predominantly through upregulation of CBG production in the liver (Wiegratz *et al.*, 2003a), which occurs to varying degrees with different estrogen components used in COCs. EE raises CBG production to a greater degree than E2V (Wiegratz *et al.*, 2003a) and estetrol (E4) (Klipping *et al.*, 2021). This implies that measures of total cortisol such as in blood or serum, may not reflect changes in bioactive free cortisol. The impact of COC on free cortisol levels is inconclusive and may be affected by COC composition (Herrera *et al.*, 2019). Nenke *et al.* (2017) observed increased CBG (both high- and low-affinity CBG) and free cortisol with COC use, but in another study increased hepatic CBG production with use of COCs containing EE/LNG was accompanied by decreased hairF and salivaryF. Increased CBG binding thus decreased free cortisol levels (Aleknaviciute *et al.*, 2017). Similar results were found after six 28-day cycle treatments of EE/DRSP, EE/LNG, or E4/DRSP, with increased total cortisol and CBG and a concomitant decrease in calculated free cortisol (Klipping *et al.*, 2021). From the above, it is clear that both the estrogen and progestin components of COCs can alter cortisol levels through different mechanisms. Furthermore, the assessment of chronic cortisol exposure

in COC users could benefit from measurements of hairF, because it represents long-term free cortisol without the influence of daily fluctuations. HairF has not been assessed in users of COCs containing EE/DRSP.

Aside from elevated free cortisol levels, negative health consequences can arise from heightened cortisol action in glucocorticoid-responsive tissues. Cortisol activity is regulated by the anti-glucocorticoid action of DHEA(S), as well as peripheral metabolism between cortisol and cortisone, via 11β-HSD enzymes. Estrogen tissue specifically alters expression of 11β-HSD enzymes. In adipose tissue, 11β-HSD1 expression is upregulated by estrogen, while in the liver and kidneys it is diminished (Mattsson & Olsson, 2007). Estrogen increases the expression of 11β-HSD types 1 and 2 in respective rat uterine tissues, and to some degree, progesterone also has an enhancing effect on 11β-HSD2 (Burton *et al.*, 1998). Decreased activity of both 11β-HSD enzymes has been found in COC users (Smith *et al.*, 1997). Furthermore, the activity of human liver 11β-HSD1 was attenuated by both EE and DRSP *in vitro* (Stapelfeld & Maser, 2017). The use of COCs containing EE/LNG, EE/DNG, EE/DRSP, and E4/DRSP is associated with decreased DHEA(S) levels. Although DNG and DRSP are antiandrogenic progestins, the rise in DHEA(S) can be partly explained by the EE-related increase in SHBG levels also found in these users (De Leo *et al.*, 2007; Klipping *et al.*, 2021; Macut *et al.*, 2015; Wiegratz *et al.*, 2003a). Interestingly, Klipping *et al.* (2021) showed that SHBG was increased significantly more and the decrease in DHEA was greater in EE/DRSP users than in users of EE/LNG and E4/DRSP. These studies, however, did not directly assess the cortisol/DHEA ratio. Cortisone and DHEA have not yet been quantified in hair of EE/DRSP-containing contraceptive users. HairE, hairDHEA, and their ratios with hairF can be used to comprehensively assess long-term cortisol activity, which may aid in clarifying the health consequences of COCs that seem to be mediated by cortisol, as well as identify users at greater risk of developing stress-related illnesses.

2.9 LC-ESI-MS/MS method

2.9.1 Overview

Liquid chromatography mass spectrometry (LC-MS) is a highly specific and sensitive analytical technique widely used for quantification of glucocorticoids and other steroids in various biomatrices. A significant advantage of LC-MS is that it offers the ability to simultaneously quantify multiple analytes. This method has been extensively used for steroid analysis in hair, because its high sensitivity makes it ideal for the quantification of low steroid concentrations (Gao *et al.*, 2016; Keevil, 2016). Immunoassay (IA), another frequently used, sensitive steroid quantification method may be a less complex and less time-consuming option. However, with this method only one steroid can be analysed at a time and the cross-reactivity that antibodies

have with some other steroids makes IAs far less selective than LC-MS (Gao *et al.*, 2016). In the various cortisol IA kits that are commercially available, this cross-reactivity leads to exaggeration of cortisol levels (Russell *et al.*, 2015). Gas chromatography (GC)-MS is another method that exists but is not commonly used for steroid analysis. Since steroids must first be derivatised to make them volatile, this method is more time-consuming than LC-MS which does not necessarily require derivatisation. Additionally, large sample volumes of hair are required for GC-MS, whereas LC-MS allows for use of smaller sample volumes (Gao *et al.*, 2016; Keevil, 2016).

The principle of LC-MS is that compounds are separated by liquid chromatography based on their chemical properties such as polarity, then detected in the mass spectrometer based on their mass-to-charge ratio (*m/z*) (Wudy *et al.*, 2018). Analytical samples are injected onto the stationary phase of the LC system. As the liquid mobile phase flows through the solid phase, analytes separate based on their interactions with mobile and stationary phases. Analytes that have a higher affinity for the mobile phase will elute first and analytes that have higher affinities for the stationary phase will elute later. As analytes elute, they enter the mass spectrometer coupled to the LC system. For analytes to be detected by the mass analyser, they must first become positively or negatively charged in the ion source (Wudy *et al.*, 2018). The two ionisation methods used for LC-MS steroid analysis in hair, electrospray ionisation (ESI) (Chen *et al.*, 2013; Ney *et al.*, 2021; Noppe *et al.*, 2015; Voegel *et al.*, 2020; Zhang *et al.*, 2013) and atmospheric pressure chemical ionisation (APCI) (Chen *et al.*, 2019; Gao *et al.*, 2013; Zhang *et al.*, 2018) are soft ionisation techniques that do not excessively fragment analytes (Gao *et al.*, 2016; Wudy *et al.*, 2018). In ESI, small charged droplets are formed by applying a high voltage to the sample and spraying it into fine droplets. The solvent is then evaporated, increasing the charge on the surface of the droplets to the extent that electrostatic repulsion results in the formation of even smaller droplets, from which ions are repelled and enter the gas phase to be detected in the mass spectrometer (Wal *et al.*, 2010). Quadrupole mass spectrometers are the most regularly employed mass spectrometers for quantification of steroids in hair and are frequently used as tandem MS (MS/MS), also referred to as a triple quadrupole. In this configuration, the first MS filters the precursor ion of a specific *m/z*, that are then fragmented in a compound-specific manner in the collision cell between the two quadrupoles. The resulting unique product ions are identified and quantified in the second MS (Keevil, 2016; Kushnir *et al.*, 2011; Wudy *et al.*, 2018). Multiple reaction monitoring (MRM) mode scans for both the precursor and product ion, which is far more selective than single ion monitoring (SIM) that identifies an analyte based only on the precursor ion (Gao *et al.*, 2016; Keevil, 2016). Further improved sensitivity and specificity of MS/MS/MS also has been exploited for cortisol and cortisone analysis in hair (Gaudl *et al.*, 2016; Quinete *et al.*, 2015).

2.9.2 Method development

An LC-MS/MS method needs to be suitable in terms of sensitivity, selectivity, and reproducibility for all analytes of interest in the intended matrix (Keevil, 2016). When selecting an LC-MS method or developing a new one, knowledge of some of the sample characteristics is important. Some of these include molecular weight, polarity, and solubility as well as the concentrations found in the biological matrix (Wal *et al.*, 2010). Based on the application, a method can be optimised for greater sensitivity or faster throughput (Keevil, 2016).

2.9.2.1 Sample preparation

When developing an LC-MS/MS method, sample preparation is an important step to consider. The sample needs to be purified to eliminate possible interference from substances that exist in the biological matrix and may co-elute with analytes of interest (Kushnir *et al.*, 2011). These interferences increase background noise and may lead to differences in the ionisation efficiency of analytes. This is known as the matrix effect and can be in the form of ion suppression or enhancement. This purification step is crucial for hair steroid analysis, since it is considered a particularly complex matrix. Another purpose of sample clean-up is to prolong the column lifetime by removing proteins that may build up over time and block the column. Sample purification can be accomplished through various methods, including protein precipitation, liquid-liquid extraction (LLE), and solid phase extraction (SPE). SPE is the sample purification method of choice for hair steroid analysis (Gao *et al.*, 2016; Wal *et al.*, 2010). Analytes are purified based on their interaction with the solid phase packing material and the aqueous phase that passes through the cartridge. Washing the cartridge with a weak solvent allows for the retention of the analytes due to a higher affinity for the stationary phase, while more polar interferences are washed out. Subsequently, analytes are eluted using a stronger elution solvent (Gao *et al.*, 2016; Wal *et al.*, 2010). Not only does SPE minimise matrix effects, but it also allows for sample concentration, making the detection of low concentration analytes easier. The choice of SPE packing material and solvents is selected based on the polarities of the analytes of interest (Keevil, 2016). The sample volume used and whether the sample should be concentrated or diluted is determined by the required sensitivity and the degree of interference from the matrix effect (Kushnir *et al.*, 2011). Additional sample preparation steps required for hair steroid analysis include washing the hair to remove external contaminants followed by extraction of steroids from the hair matrix (Gao *et al.*, 2016).

2.9.2.2 Liquid chromatography

The LC part of the method is developed to achieve separation of analytes from matrix components and each other. Good separation is characterised by sharp, non-overlapping peaks

on the chromatogram. The selection of stationary and mobile phases has the greatest contribution to separation efficiency (Wal *et al.*, 2010). The hydrophobicity of steroids allows for successful separation by reversed-phase columns, and most LC-MS methods utilise octadecylsilane (C18) columns to separate cortisol, cortisone, and other steroids (Gao *et al.*, 2016). Biphenyl columns are generally used for isomers or very closely related structures (Lindner *et al.*, 2017). The particle size of the stationary phase greatly affects separation, with smaller particles enabling better peak resolution. The mobile phase is made up of two parts: the aqueous phase (phase A) and the organic phase (phase B), which is usually methanol or acetonitrile The ratio of phase A to phase B can be isocratic or the ratio can be changed over time to create a gradient for better separation of analytes when there are many compounds to separate. The pH of the mobile phase often needs to be controlled for ideal peak shapes and to ensure that results are repeatable (Wal *et al.*, 2010). Mobile phase additives such as formic acid, ammonium formate, or ammonium acetate are usually used for this purpose. However, for steroids, their main purpose, which will be discussed further on, is to enhance ionisation (Marcos & Pozo, 2016). Parameters such as temperature and flow can be adjusted and optimised not only to improve resolution, but also to reduce run time. Increasing flow rate and temperature reduces run time, while decreasing the flow rate improves separation. The use of shorter columns with a smaller diameter can also reduce run time and solvent use (Wal *et al.*, 2010).

2.9.2.3 Ionisation

Sensitive detection by MS can be achieved by selecting the appropriate ionisation polarity along with a suitable mobile phase modifier. Based on structure, some analytes are more suited to positive ionisation mode and others to negative mode (Keevil, 2016; Marcos & Pozo, 2016). Mobile phase modifiers such as ammonium formate and formic acid aid in positive ionisation, while ammonium acetate aids in negative ionisation (Keevil, 2016; Wal *et al.*, 2010). Ammonium fluoride can be used for both positive and negative ionisation depending on the analyte structure. For example, it can improve the sensitivity for glucocorticoids and androgens in positive mode, whereas for estrogens it aids in negative ionisation (Gaudl *et al.*, 2016). Even with the aid of mobile phase modifiers in improving ionisation, DHEA has poor ionisation efficiency. As a result, the majority of LC-ESI-MS/MS methods use derivatisation to enhance sensitivity for detection of the low concentrations in hair (Dong *et al.*, 2015; Hobo *et al.*, 2020). Derivatisation entails the addition of a more easily ionisable group to the analyte structure (Higashi & Ogawa, 2016; Santa, 2013). Derivatisation with picolinic acid is used for phenolic steroids such as cortisol, cortisone, and estrogens (Higashi & Ogawa, 2016; Marcos & Pozo, 2016). Keto groups of androgens and glucocorticoids can be derivatised with Girard reagent T, Girard reagent P, hydrazino-1-methylpyridine (HMP), and 2-hydrazinopyridine (2-HP) (Dong *et*

al., 2015; Higashi & Ogawa, 2016; Santa, 2013). Derivatisation of carbonyl groups with 2-HP was shown to enhance the response of progestogens and androgens (testosterone, progesterone, pregnenolone, androstenedione) in positive ESI mode at least twice as much as with HMP derivatisation, while the enhancement of DHEA response was comparable between 2-HP and HMP. Derivatisation with either 2-HP or HMP resulted in 1600-fold improved sensitivity of DHEA compared to no derivatisation (Higashi *et al.*, 2007). Although 2-HP derivatisation has not been used for the quantification of steroids in hair, it could have the potential to improve the detection of low steroid levels in this matrix.

2.9.2.4 Quantification

Analytes are quantified by adding a known amount of an internal standard (IS). The relationship between the relative intensities of the analyte and IS is used to calculate the concentration of the analyte (Wal *et al.*, 2010). IS used for quantification should have chemical characteristics that are very similar to those of the analytes. Stable isotopes labelled with deuterium (d) or ^{13}C are ideal internal standards because they have similar chemical properties to the unlabelled analyte, thus their extraction recovery and chromatographic separation will be similar. Nevertheless, their *m/z* differs, allowing for mass spectrometric differentiation between the two (Wudy *et al.*, 2018). Since the IS is subjected to the same steps as the analyte, including sample preparation, chromatographic separation, and ionisation, the use thereof accounts for sample loss during preparation and to some degree normalises for matrix effects (George *et al.*, 2018; Hobo *et al.*, 2020). An additional advantage of stable isotopes is that they enhance the sensitivity of analytes that are present in low concentrations by the carrier effect. Instead of the small amounts of analyte that would have been lost by adsorption, small amounts of isotope are lost instead, leaving enough analyte to be detected (Rychlik, 2011).

2.9.3 Method validation

Once an LC-MS/MS method has been developed and before it can be implemented, validation is crucial to ensure that the method exhibits adequate performance and is suitable for the intended purpose (Honour, 2011). The process of method validation investigates the degree to which measurement may be influenced by variables in the environment, matrix, and analytical procedures (Wal *et al.*, 2010). Method validation for LC-MS should include assessment of selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, and stability. The selectivity of the method is its ability to distinguish between the analyte and other compounds present. The sensitivity is evaluated by the limit of detection (LOD) and the Lower Limit of Quantification (LLOQ). The LOD is the lowest concentration that can be detected and is usually defined by a signal-to-noise ratio (S/N) of 3, while the LLOQ is the lowest concentration that can be accurately and precisely measured and is defined by an S/N of 10. The linearity is the

degree to which the measurements obtained by the method are proportional to the concentration of the analytes within a defined range. The accuracy is how well the calculated concentration of the results agrees with the known concentration. Precision indicates that results are reproducible when repeated within and between runs. The recovery gives an indication of how efficient the extraction is. The recovery need not be 100% but should be repeatable for all analytes. The matrix effect should be determined to avoid false results by interference from other compounds. The chemical stability of analytes in the biological matrix should be determined over given time periods under certain conditions to avoid variations in analyte behaviour (Honour, 2011; Wal *et al.*, 2010).

CHAPTER 3 METHODS AND MATERIALS

3.1 Materials

3.1.1 Solvents, Chemicals and Analytical standards

High performance liquid chromatography grade solvents including water (365-4), methanol (230-4), isopropyl alcohol (323-4), acetonitrile (017-4), and acetone (010-4) were purchased from Honeywell, Burdick & Jackson, supplied by Anatech, South Africa. Ammonium fluoride (216011), formic acid (94318), ammonium formate (70221), and 2-hydrazinopyridine (H17082) were purchased from Sigma AldrichCo (St. Louis MO, USA). The following standards and deuterated standards were purchased from Merck KGaA (Darmstadt, Germany): cortisol (C-106), cortisone (C2755), DHEA (D4000) cortisol-d_4 (C-113), cortisone-d_8 (900170). 5-Androsten-3β-ol-17-one-16-d_2 (DHEA-d_2) (A8500-009) was purchased from Steraloids Inc.(Newport RI, USA).

3.1.2 Instrumentation/equipment

An MM400 Retsch ball mill, Phenomenex SPE column vacuum manifold (AHO-6024), purchased from Separations (Johannesburg, South Africa), and Bond Elut C18 SPE cartridges from Agilent (12102118), were used for sample preparation. The LC system consisted of an Agilent 1290 Infinity equipped with a binary pump (G4220A), vacuum degasser (G1330B), thermostat autosampler (G4226A), temperature-controlled column compartment (G1216C) and an Agilent Eclipse Plus C18 RRHD (1.8 µm, 2.1 mm x 50 mm) column (959757-902) and Eclipse plus C18 guard column (1.8 µm, 2.1 x 5 mm). This was coupled to an Agilent 6460 triple quadrupole mass spectrometer (G6460A), with a jet stream ESI source. All Agilent instruments were purchased through Chemetrix (Midrand, South Africa) from Agilent Technologies, (Santa Clara CA, USA). Agilent MassHunter workstation software, including LC/MS Data Acquisition software for 6400 series triple quadrupole (B.07.01), Quantitative Analysis software (B.06.00) and Qualitative Analysis for QQQ software (B.06.00), was used for data acquisition and analyte quantification. Optimisation of ionisation conditions was performed using Agilent technologies MassHunter workstation software Optimiser for 6400 series triple quadrupole (B.07.01) and Source and iFunnel Optimiser for 6400 triple quadrupole (B.07.01).

3.2 Preparation of stock standard solutions

Standard stock solutions of all analytes and stable isotopes were prepared in methanol at 100 ppm (µg/ml), from which 10 ppm and 1 ppm were prepared by further dilution with methanol. Stock solutions were stored at -20°C until use.

3.3 Preparation of working solution and calibration solutions

For method development stock standard solutions were diluted with methanol to the specified concentration. A mixture of cortisol, cortisone, and DHEA standards (160 ppb (ng/ml), 320 ppb, and 160 ppb respectively) was prepared, which was further diluted and then added to methanol or blank hair matrix (Section 3.6) to obtain the calibration and quality control standards. A mixture of stable isotopes was prepared for use as internal standards at the following concentrations, 10 ppb cortisol-d_4, 20 ppb cortisone-d_8, and 50 ppb DHEA-d_2.

3.4 Sample collection

Ethical approval was obtained from the Health Research Ethics Committee (HREC) of the North-West University (NWU), for the use of samples previously collected for the Estrogen, Biotransformation, and Oxidative Stress Status (eBOSS) study (NWU-00344-16-A1) in this sub-study (NWU-00271-21-A1). Participants were recruited between April 2017 and October 2019 by advertisements placed on the NWU's social platforms, as well as in shopping centres, doctors' offices, and pharmacies in Potchefstroom. As indicated by power calculations the minimum required sample size was 20. Before sample collection, all participants consented to the use of their samples by signing informed consent forms. Hair samples were collected from Caucasian women using COCs; Caucasian women not using any COCs; and Black African women not using COCs. Participants were aged 18-35, had hair longer than 3 cm, and had been using COCs containing 3 mg DRSP and 0.03 mg or 0.02 mg EE for at least 3 months. Exclusion criteria were: previous use of other COCs or hormones in the past 4 years (non-users); irregular menstrual cycle; pregnancy or breastfeeding in the past 6 years; excessive smoking (more than 5 cigarettes a day); BMI above 30; and presence of any chronic disease (including diabetes, asthma, liver or kidney disease) or use of chronic medication (Venter *et al.*, 2021).

For method development and validation, additional samples were collected in December 2021 from 10 Caucasian and 10 Black African women (NWU-00271-21-A1). For these volunteers, hair had to be 3 cm or longer, but there were no other inclusion or exclusion criteria.

Hair from participants and volunteers was tied with string and cut with scissors close to the scalp at the posterior vertex of the head (Gao *et al.*, 2016). Hair growth is constant at this position of the head, and hair from this region shows lower variation of cortisol and cortisone than hair from other head regions (Zhang *et al.*, 2013). Samples were stored at room temperature protected from light until analysis, since ultraviolet light causes steroid degradation, and storage at -20°C may cause swelling of the hair, leading to loss of steroids (Gao *et al.*, 2016).

3.5 Sample preparation and LC-ESI-MS/MS method

The final sample preparation and LC-ESI-MS/MS method as developed and optimised in the following chapter is now briefly described.

The first 3.3 cm of hair from Caucasians and 2.7 cm from Black Africans (Loussouarn *et al.*, 2016) was cut and washed in 3 ml isopropanol for 3 minutes to remove external contaminants, then allowed to dry in a fume hood for at least 12 hours. Once completely dried, extraction of cortisol, cortisone, and DHEA from 20 mg of hair was performed by 18-hour incubation, while rotating, in 1 ml methanol and 50 µl IS mixture. The methanol containing the extracted analytes was collected and stored at -80°C for 1 hour, then centrifuged at 10,000 rpm for 15 min to precipitate proteins. The supernatant was dried at 55°C under flow of nitrogen and resuspended in 1 ml cold acetonitrile containing 0.1% formic acid, then kept at -20°C for 30 min before further protein precipitation by centrifugation under the same conditions as described for methanol above. The supernatant was again dried at 55°C and the residue resuspended in 300 µl methanol and 1 ml water for SPE. The resuspended samples were loaded onto Bond Elut C18 SPE cartridges (Agilent) that had been conditioned and equilibrated with 4 ml acetone followed by 4 ml water. The cartridge was washed with 3 ml water containing 20% (v/v) methanol to remove unwanted impurities. Analytes were eluted with 2.4 ml acetone, dried under nitrogen, and resuspended in 200 µl of starting mobile phase (40% methanol, 60% water), which was filtered through 0.2 µm Nylon Spin-X filters.

The mobile phases for liquid chromatography consisted of 1 millimolar (mM) ammonium fluoride in water as mobile phase A, and 1 mM ammonium fluoride in methanol and 0.4% (v/v) water as mobile phase B. The chromatographic gradient was kept at 40% B for 3 min, gradually increased to 61% at 8 min, 65% at 14 min, and to 100% at 15 min. An injection volume of 10 µl was used.

3.6 Preparation of blank samples and quality control samples

Distal hair sections past 12 cm from the scalp were used from 8 individuals (Chen *et al.*, 2013). The hair was washed and cut into 3 cm sections of which each 20 mg was separately incubated as described above. The fluid from all sections was pooled before protein precipitation. The pooled blank was aliquoted into separate tubes to represent 20 mg of hair, dried under nitrogen at 55°C, and stored at -20°C until use. The quality control (QC) samples were prepared by spiking pooled blank samples with the required volume of master mix.

CHAPTER 4 METHOD DEVELOPMENT

Various LC-ESI-MS/MS methods have been established to simultaneously quantify cortisol, cortisone, and DHEA in hair. Due to the low concentrations of these steroids in hair and poor ionisation efficiency of DHEA, these methods generally require derivatisation to enhance sensitivity (Dong *et al.*, 2017; Hobo *et al.*, 2020). In this study, it was attempted to develop a non-derivatised method to avoid unnecessary time-consuming derivatisation steps. Non-derivatised LC-ESI-MS/MS methods that quantified cortisol, cortisone, and DHEA in other biomatrices were tested and further developed and optimised to achieve the sensitivity required to measure the low levels of these hormones in hair. Similarly, an LC-ESI-MS/MS method using 2-HP derivatisation was also developed from methods that quantified the analytes in other biomatrices. The two methods were compared for sensitivity and selectivity.

4.1 Non-derivatised method

4.1.1 Mass spectrometry

4.1.1.1 Mobile phase modifiers and MRM conditions

The first step of the method development process is to ensure sensitive detection of analytes by the MS. Selection of mobile phase modifiers greatly contributes to the sensitivity of an LC-ESI-MS method. In an Agilent application note, Juck and Long (2017) investigated various mobile phase modifiers for LC-ESI-MS/MS quantifying serum levels of progesterone, corticosteroids, estrogens, and some androgens in both positive and negative mode. They found that the use of ammonium fluoride resulted in greater responses for all steroids, including cortisol and cortisone in positive mode, compared to ammonium hydroxide or formic acid. Furthermore, for a similar panel of steroids, ammonium fluoride compared to ammonium formate yielded improved sensitivities for cortisol, cortisone, and DHEA (Lindner *et al.*, 2017). Ammonium fluoride has been used to enhance the sensitivities of hairF and hairE (Gaudl *et al.*, 2016), but its effect on hairDHEA sensitivity has not yet been investigated. Son *et al.* (2020) showed that when tested against formic acid or ammonium formate alone, a combination of formic acid and ammonium formate improved signal intensities of salivary cortisol, cortisone, and DHEA. Therefore, ammonium fluoride and a combination of formic acid and ammonium formate were tested as mobile phase modifiers in this study. Mobile phase modifiers were investigated by comparing the abundances of precursor and product ions obtained for each analyte from two different mobile phase compositions. The first composition consisted of 0.01% (v/v) formic acid and 1 mM ammonium formate in water as mobile phase A and 0.01% (v/v) formic acid and 1 mM ammonium formate in methanol as mobile phase B (Son *et al.*, 2020). For the second composition, mobile phase A was 1 mM ammonium fluoride in water and mobile phase B was

methanol (Juck & Long, 2017). This was performed through 1 µl direct infusion of individual 1 ppm standards and stable isotopes into the ionisation source of the MS with mobile phase ratios of 50% phase A to 50% phase B. Ionisation was in positive mode ESI for all analytes and source conditions were as in an Agilent technologies application note for steroid analysis (Juck & Long, 2017), which were as follows: gas temperature of 250 °C, gas flow of 11 L/min, nebulizer pressure of 35 psi, sheath gas flow 11 L/min, capillary voltage of 3,000 V and nozzle voltage of 0 V. Agilent MassHunter Optimiser software was used to optimise the MRM conditions for both mobile phase compositions. The most abundant precursor and product ions were determined, and the fragmentor voltages and collision energies required for their formation were optimised. The relative intensities of each analyte with both mobile phase modifiers, can be seen in Figure 4-1. Fragmentor voltages were optimised over a course range from 50 – 200 V, while collision energies were optimised from 0 – 50 eV.

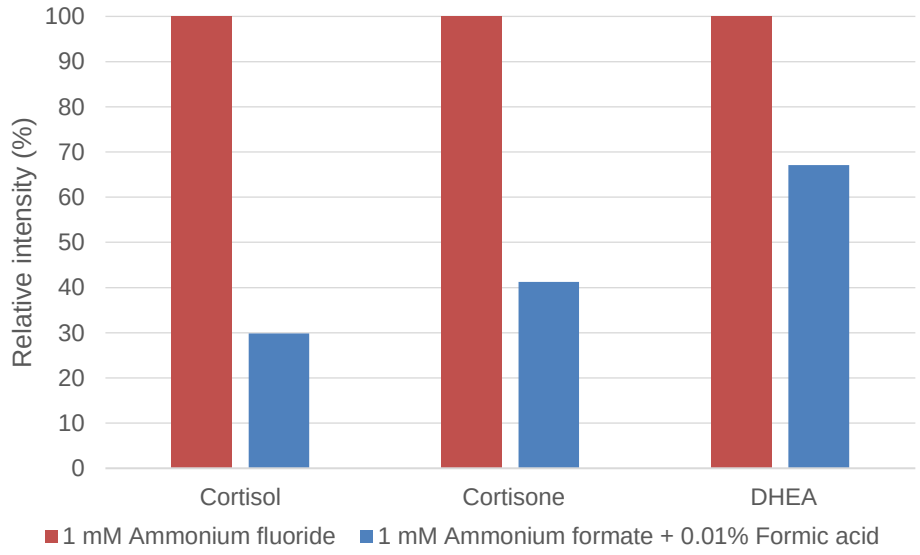


Figure 4-1: Comparison of the relative intensities for the different mobile phase modifiers. Intensities of each analyte are expressed as the percentage relative to the product ion abundance with 1 mM ammonium fluoride as mobile phase modifier.

Both mobile phase compositions sufficiently produced precursor and product ions for all analytes, 1 mM ammonium fluoride yielded more abundant product ions for all analytes than 0.01% formic acid and 1 mM ammonium formate. Therefore, the mobile phase composition used onward consisted of 1mM ammonium fluoride in water as mobile phase A and methanol as mobile phase B. The optimal MRM conditions for each analyte are displayed in Table 4-1**Error! Reference source not found.**. Protonated molecular ions $[M + H]^+$ were selected as precursors for all analytes. Similarly to Gao *et al.* (2013), Lindner *et al.* (2017), and Son *et al.* (2020), precursor ions for DHEA that formed due to water loss $[M - H_2O + H]^+$ were the most abundant. Nonetheless, $[M + H]^+$ was later selected due to lower background noise, leading to improved sensitivity.

Table 4-1: Optimised MRM conditions showing precursor ions and the most abundant product ions, as well as fragmentor and collision energies for each analyte and stable isotope.

Compound	Molecular formula	Molecular weight (g/mol)	Fragmentor energy (V)	Precursor Ion (m/z)	Collision energy (eV)	Product ion (m/z)
Cortisol	$C_{21}H_{30}O_5$	362.5	127	363.2	28	121.1
Cortisone	$C_{21}H_{28}O_5$	360.4	127	363.2	24	163
DHEA	$C_{19}H_{28}O_2$	288.4	81	289.2	12	253.1
Cortisol-d_4	$C_{21}D_4H_{26}O_5$	366.5	148	367.2	24	121.1
Cortisone-d_8	$C_{21}D_8H_{20}O_5$	368.4	124	369.2	24	169.1
DHEA-d_2	$C_{19}D_2H_{26}O_2$	290.4	70	291.2	8	255.2

4.1.1.2 Source conditions

Source conditions were optimised using Agilent MassHunter Source and iFunnel Optimiser software. This was performed by 1 µl direct infusion of individual 1 ppm standards in MRM acquisition mode using the MRM conditions selected for each analyte as displayed in Table 4-1**Error! Reference source not found.**. The source conditions of the direct infusion method described above were used as a starting point and the software was used to change one parameter at a time while keeping the remainder constant. The parameters investigated can be seen in Table S 1, which shows the steps and the ranges in which they were tested. The optimal setting for each parameter was selected by comparing the abundances of the analytes for each parameter. During this process, DHEA was prioritised, due to its low abundance compared to the other analytes.

The optimal source conditions for the analytes are summarised in Table 4-2**Error! Reference source not found.**. Although source conditions were optimised to specifically enhance the ionisation of DHEA, due to its low abundance, the source conditions selected were also ideal for enhancement of cortisol and cortisone ionisation.

Table 4-2: Optimised source conditions for all analytes.

Capillary voltage (V)	Drying gas flow (L/min)	Drying gas temperature (˚C)	Nebulizer (psi)	Nozzle voltage (V)	Sheath gas flow (L/min)	Sheath gas temperature (˚C)
3500	12	150	40	0	10	400

4.1.2 Liquid chromatography

The LC method was developed by injecting 1 µl of a mixture containing 1 ppm standards of each analyte and using the optimised MRM and source conditions as summarised in Table 4-1 and Table 4-2 respectively. Initially, the chromatographic conditions as used by Juck and Long (2017) were tested. The flow rate was 0.4 ml/min with a column temperature of 40 °C. The gradient applied started at 40% mobile phase B and gradually increased to 60% over 9 minutes, then increased to 95% from 9-11 minutes. These conditions were then adjusted to achieve optimal separation of analyte peaks and to shorten the method. The chromatogram of the preliminary separation method (Table S 2) is shown in Figure 4-2. The retention times from this method were used to identify analyte peaks during further optimisation.

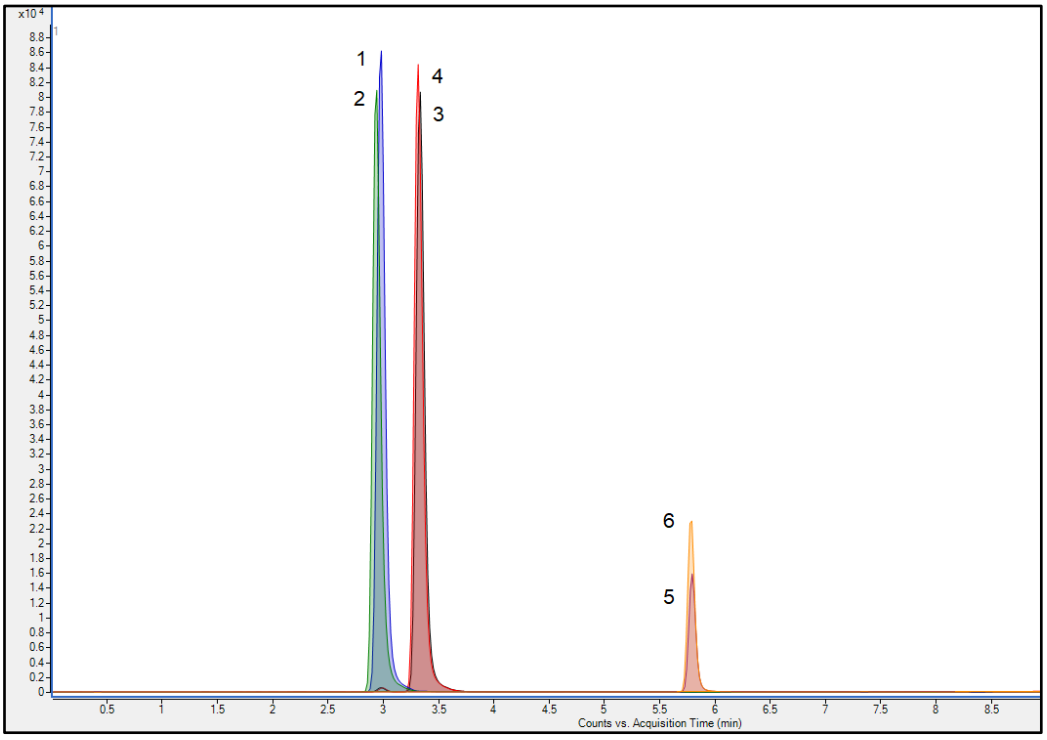


Figure 4-2: Chromatogram of preliminary chromatograpic separation of analytes. 1. Cortisone, 2. Cortisone-d8, 3. Cortisol, 4. Cortisol-d4, 5. DHEA, 6. DHEA-d2.

Later on, after the sample preparation method (Section 4.1.3) and injection volume (Section 4.1.4) were optimised, 10 µl injections of medium concentration spiked blank matrix were used to further adjust chromatographic separation to separate the analytes of interest from other closely eluting compounds that could result in interference (Keevil, 2016). After the last analyte had eluted, the mobile phase was kept at 100% B for 10 minutes while scanning for all masses in the MS to determine the time required to completely remove more non-polar compounds that may remain in the column. The post run time, the time at which the mobile phase is kept constant after the analytical run, was evaluated to ensure that the column is equilibrated and that the binary pump pressure is stabilised between runs.

The gradient for the final method is shown in Table 4-3. The flow rate was 0.2 ml/min and the column temperature was 40° C. Analyte retention times were confirmed by injection of individual standards and can be seen in Table 4-4. As seen in Figure 4-3, all analytes were sufficiently separated from neighbouring peaks in spiked hair. Seven minutes at 100% B was required to remove more polar compounds from the column, while three minutes post time was sufficient for binary pump pressure to return to starting pressure.

Table 4-3: The liquid chromatographic mobile phase gradient indicating the times over which the percentage mobile phase B changes. The flow rate was 0.2 ml/min and the column temperature was 40° C.

Time (min)	% Mobile phase B
0	40
3	40
8	61
14	65
15	100
22	100
23	40

Table 4-4: The retention times of each analyte for the final chromatographic method.

Analyte	Retention time (min)
Cortisol	7.97
Cortisol-d_4	7.95
Cortisone	7.24
Cortiosone-d_8	7.16
DHEA	12.19
DHEA-d_2	12.19

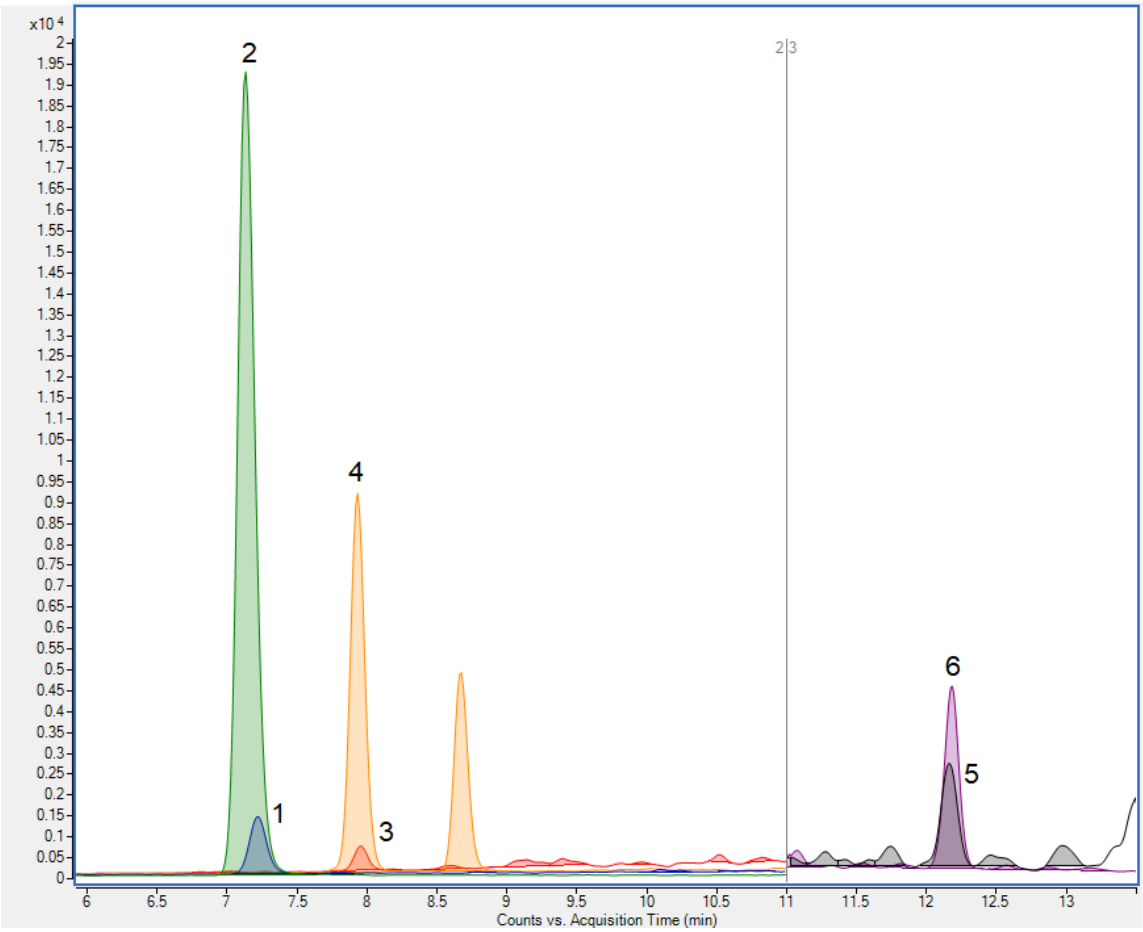


Figure 4-3: Chromatogram showing the separation of analytes in a spiked blank hair sample. 1. cortisone, 2. Cortisone-d8, 3. Cortisol, 4. cortisol-d4, 5. DHEA, 6. DHEA-d2.

4.1.3 Sample preparation

Due to visible differences in hair texture and participant-reported differences in hair treatment, the ethnicity of volunteers was considered during development of sample preparation to ensure accommodation for variances in matrix components and extraction efficiency.

4.1.3.1 SPE

Sample clean-up is required to reduce interference from matrix components that may block analytical columns, contaminate the mass analyser and supress ionisation (Gao *et al.*, 2016). The most commonly used sample clean-up method for hair steroid analysis is SPE using reversed phase C18 columns (Chen *et al.*, 2019; Raul *et al.*, 2004; Zhang *et al.*, 2018). The goal during SPE development was to sufficiently remove interfering matrix components, yet maximally recover analytes of interest.

For sample clean-up, the SPE method by Chen *et al.* (2019) was evaluated and optimised. The SPE method was optimised using a mixture of standards and stable isotopes. The final concentrations of cortisol, cortisone, DHEA and their respective stable isotopes were 10 ppb, 20

ppb and 50 ppb, respectively. These concentrations were close to the highest reported physiological values, except for the concentration of DHEA that was chosen at 10 times the highest physiological value (Chen *et al.*, 2019; Gao *et al.*, 2013) to enhance detection by the carrier effect (Kataoka *et al.*, 2013; Petersen & Vouros, 1977; Wooding *et al.*, 2015). The authors used Pro Elut SPE C18 columns from Dikma Technologies Inc., but in the present study similar SPE cartridges, Bond Elut C18 were used due to availability. In the initial method the column was conditioned by 4 ml methanol followed by 4 ml of water. The standards were dissolved in 100 µl methanol (MeOH) and 1 ml water, which was loaded onto the SPE column. The column was washed with 3 ml water to remove impurities and analytes were eluted with 800 µl methanol (Chen *et al.*, 2019). Conditioning and loading were performed under gravity flow. For washing and elution, gravity flow was used until the flow stopped, whereafter a vacuum was applied to ensure that all wash solvent was removed and that all eluent was collected. The eluent was evaporated at 55 °C under flow of nitrogen and resuspended in 200 µl of the initial mobile phase for LC-MS/MS analysis (Chen *et al.*, 2019), which was then filtered through 0.22 µm Nylon Spin-X filters by centrifuging at 4,000 rpm for 2 min to remove particulates from the SPE cartridges that may block the LC column. Different loading solutions were tested with varying methanol volumes. The elution volume was assessed by separately collecting the eluent from one elution of 800 µl and a second elution of the same volume. Both eluents were analysed to determine whether all analytes were collected in the first elution step or if a larger elution volume was required. Subsequently, different elution volumes and solvent combinations of methanol and a stronger eluent, acetone, were then tested to maximise analyte recovery. The wash step was collected and analysed to determine whether or not analytes were lost. Analyte recoveries were determined by comparing the mass spectrum abundances of each method to the response obtained from standards of the same concentrations that were not subjected to SPE. A loading solvent consisting of 300 µl methanol and 1 ml water yielded the highest analyte recoveries. As determined by analysing the collected loading solvent, analytes were not lost in this step. Analysis of the second elution volume revealed that 800 µl was insufficient to completely elute analytes of interest, and the eluent that yielded the highest analyte recovery was 2.4 ml acetone. The results of each step tested can be viewed in the supplementary materials (Figure S 1 and Figure S 2). Consequently, methanol in the conditioning step was replaced with 4 ml acetone, as recommended by the SPE cartridge suppliers regarding the use of a stronger elution solvent such as acetone.

After extraction methods were tested, it was observed that stable isotope responses were considerably lower in samples compared to responses of the same concentrations in standard solution (Figure S 3), making it apparent that components in the hair matrix may have led to ion suppression. To increase clean-up of these components, stronger wash solvents were tested, including 20% and 30% methanol in water. A 20 mg sample from one Caucasian and one Black

African volunteer were used for each wash solvent tested. The responses of the analytes for each wash solution tested can be seen in Figure 4-4. As seen in full scans of eluents (Figure S 4), background was reduced when washing with 20% and 30% methanol compared to water alone, indicating that sample clean-up of co-eluting compounds was improved. Together with this, increased cortisol and cortisone responses from 20% methanol and decreased responses from 30% methanol compared to 100% water suggests that 20% methanol reduces ion suppression, but 30% methanol leads to analyte loss. However, changing the washing solvent had little effect on DHEA responses indicating that none of the wash solvents were strong enough to elute DHEA or improve cleanup of components that coeluted with this analyte. Considering the results above, 20% methanol was chosen as the wash solvent. The final SPE procedure is summarised in Figure 4-5.

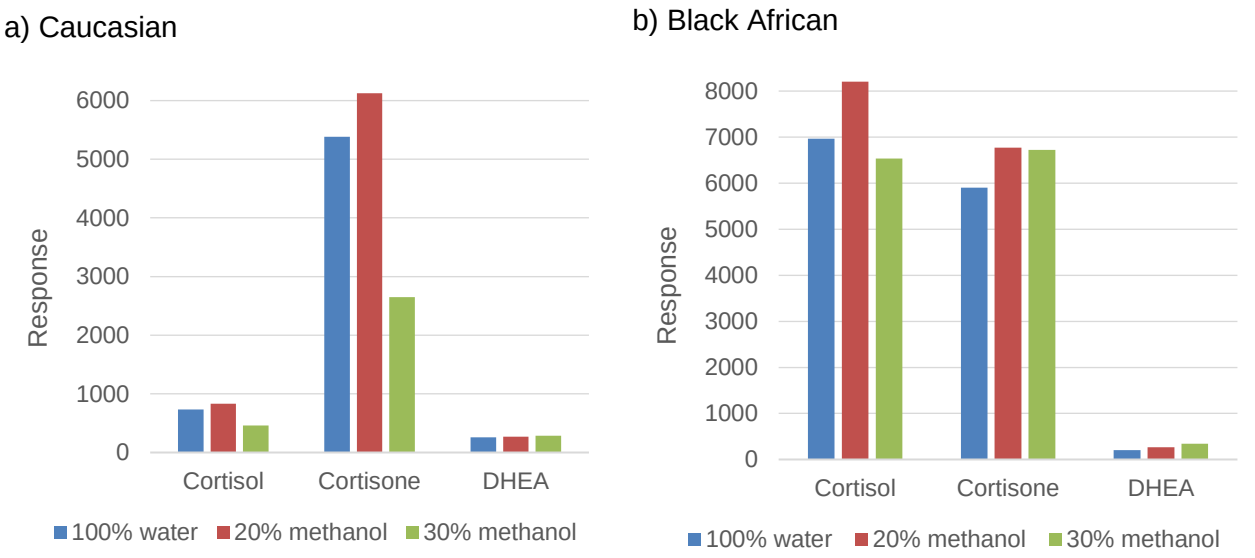


Figure 4-4: Visual illustration of the responses of each analyte for the SPE wash solutions tested using a a) Caucasian and a b) Black African hair sample.

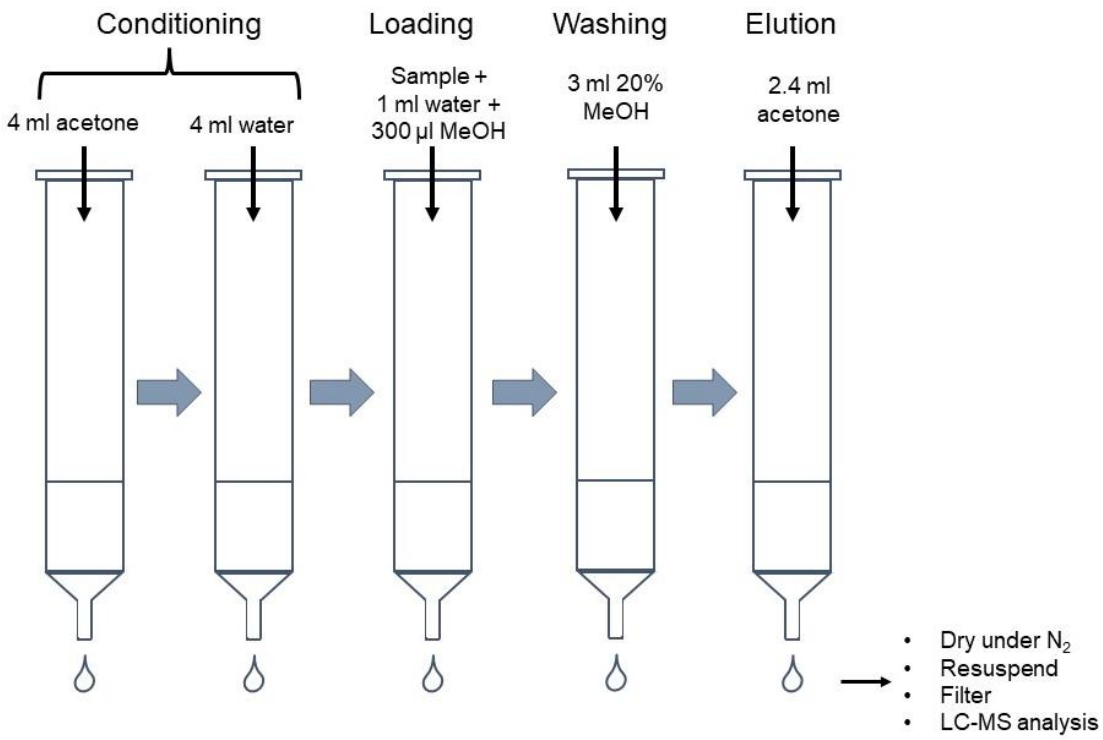


Figure 4-5: Schematic diagram of the final SPE method.

4.1.3.2 Steroid extraction from hair

In order to analyse steroids by LC-MS they need to be extracted from the hair strands and dissolved in solution. Methanol is the most ideal extraction solvent, because of its ability to dissolve hydrophilic compounds (Gao *et al.*, 2016). Incubation time for extraction is important to consider and is largely dependent on the preparation procedure (Xue *et al.*, 2013). Hair is either ground into powder, minced with scissors or used whole. Milling or grinding of hair is thought to be necessary for effective steroid extraction by releasing glucocorticoids from the hair matrix and increasing surface the area for solvent interaction. However, the milling process may lead to sample loss and steroid degradation by the heat produced (Gao *et al.*, 2016; Greff *et al.*, 2019). This can be avoided by the use of whole hair, which may require longer incubation times for complete extraction of certain steroids such as cortisone and DHEA (Chen *et al.*, 2013; Zhang *et al.*, 2013). Milled and non-milled extraction methods were compared in this study to maximise steroid extraction.

To test methods for extracting steroids from hair strands, hair from one Caucasian and one Black African volunteer was used. The proximal 6 cm was measured and cut to ensure that there was enough to test each method in triplicate. Hair was washed in 3 ml isopropanol for 3 minutes to remove any external contaminants including steroids from sweat or sebum that may be deposited on the outside of the hair then left to dry in a fume hood for at least 12 hours (Gao *et al.*, 2016; Xue *et al.*, 2013). Once dried, 20mg of hair was carefully weighed and cut into ~1

cm segments. Methanol (1 ml) and 50 µl IS mix were added to each sample. For method A, 3 x 3 mm stainless steel balls were added and hair was milled for 5 min at 30 Hz using a Retsch MM 400 ball mill. The balls were then removed, and the milled hair was incubated for 18 hours while rotating. In method B and C, nonmilled hair was incubated for 18 and 24 hours, respectively (Dong *et al.*, 2017; Gao *et al.*, 2013). After incubation the milled samples were centrifuged at 14,000 rpm for 10 minutes. Centrifugation was not necessary for non-milled samples because the liquid could easily be removed from hair snippets. The supernatant from milled samples and the liquid from non-milled samples were dried under flow of nitrogen as described in Section 4.1.3.1 before resuspension for SPE.

The analyte concentrations extracted were calculated relative to the mass of hair using the ratio of analyte response to IS response to account for sample loss during preparation. At first 1 µl was used, but when responses appeared very low, the injection volume was increased to 10 µl. Calculation of concentrations in Black African samples was not possible due to co-eluting compounds that could not be separated. The mean analyte concentrations obtained can be seen in Table 4-5. For cortisol and cortisone, the highest concentrations were obtained from milled hair, while non-milled hair incubated for 18 hours gave the highest concentration of DHEA. The concentrations obtained from non-milled hair were comparable for 18 hours and 24 hours. Additionally, when milling the hair in methanol, less sample was lost and less heat was generated than milling before methanol was added, addressing the problem of sample loss described by Dong *et al.* (2017). The higher concentrations of most analytes from milled hair did not appear to significantly improve sensitivity, and as a result non-milled hair incubated for 18 hours was selected as the final extraction method for time efficiency.

Table 4-5: Mean Analyte concentrations obtained from Caucasian and Black African hair samples for each extraction method tested. Analyte concentrations were calculated using the ratio of analyte response to IS response and are expressed relative to the mass of hair weighed.

Analyte	Milled and incubated for 18 hours	Non-milled and incubated for 18 hours	Non-milled and incubated for 24 hours
Cortisol (pg/mg)	2.8	2.4	2.1
Cortisone (pg/mg)	6.8	5.9	5.6
DHEA (pg/mg)	30.4	32.9	29.0

4.1.3.3 Protein precipitation

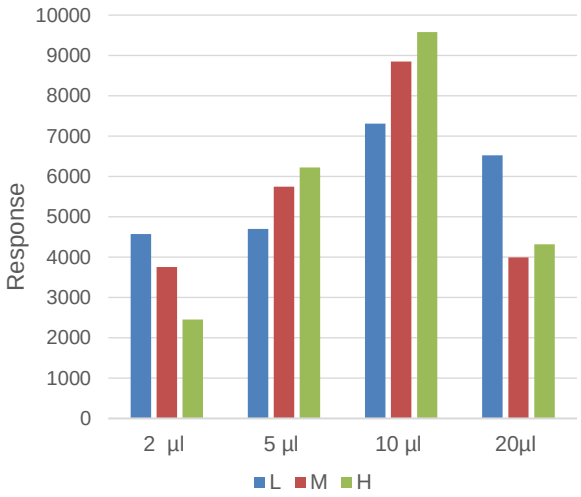
Most sample preparation methods for LC-MS analysis of hair steroids do not include a protein precipitation step (Binz *et al.*, 2016; Dong *et al.*, 2017; Hobo *et al.*, 2020; Noppe *et al.*, 2015).

Therefore, this was not considered until aggregated proteins were visible during preparation of the pooled blank sample for method validation. Two protein precipitation steps were then incorporated into the sample preparation process. After incubation, 2 ml of methanol was added to the liquid that was removed from the hair snippets. This was frozen at -80°C for 1 hour and then centrifuged at 4,000 rpm, 0°C, for 20 minutes. Next, the supernatant was dried at 55°C under flow of nitrogen and the dried residue was resuspended in 3 ml acetonitrile with 0.1% formic acid, which was then frozen, centrifuged and dried as described above for methanol (Baier *et al.*, 2020; de la Torre *et al.*, 2021; Polson *et al.*, 2003). The dried residue was resuspended for SPE.

4.1.4 Further optimisation

Next, to find the optimal injection volume 2 µl, 5 µl, 10 µl and 20 µl were tested. Each volume was tested using blank hair matrix spiked at low, medium, and high concentrations. The responses of each concentration and injection volume are shown in Figure 4-6. In most cases the response increases with increasing injection volume, except for cortisol that decreases from 10 µl to 20 µl indicating that there is ion suppression when 20 µl is injected. As a result, 10 µl was chosen as the ideal injection volume.

a) Cortisol



b) Cortisone

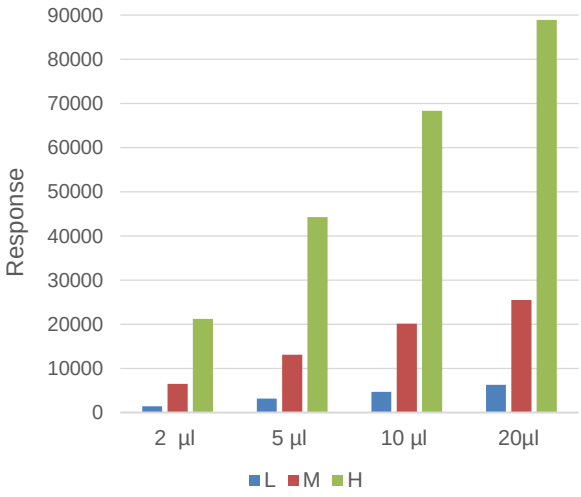


Figure 4-6: Visual representation of responses obtained from the injection volumes tested at three analyte concentration levels (L: low, M: medium, and H: high).

c) DHEA

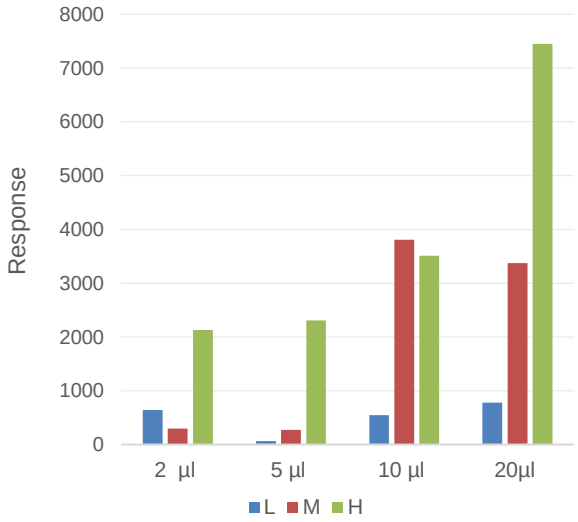


Figure 4-6 Continued: Visual representation of responses obtained from the injection volumes tested at three analyte concentration levels (L: low, M: medium, and H: high).

Until this point, only mobile phase A contained ammonium fluoride, so there is less mobile phase modifier to aid in the ionisation of analytes that elute at a higher percentage of mobile phase B. Therefore, ammonium fluoride was also added to mobile phase B to determine if analyte responses improved. This was tested using the same spiked samples as for injection volume. Responses were increased for all concentrations, which led to the final method consisting of water with 1 mM ammonium fluoride as mobile phase A and methanol with 0.4% water and 1 mM ammonium fluoride.

Finally, the first 2.5 minutes before the first analytes eluted, as well as from 11 minutes onwards, after the last analyte had eluted, was set to go to waste so that compounds that are not of interest would not enter the MS and affect ionisation and detection of analytes. Furthermore, time segments were added in which the MS was set to only analyse cortisol and cortisone from 2.5 min to 9 min and DHEA from 9 min to 11 min. This enabled a longer dwell time for each analyte, which is the time that the MS scans for each ion in a cycle. Dwell times were optimised to improve detection of analytes that had lower abundances. Within the time segments a dwell time of 200 ms was the best for each analyte and stable isotope. Although the addition of time segments had little effect on cortisol and cortisone, background was reduced for DHEA, lowering the baseline and improving integration for quantification (Figure S 5 and Figure S 6).

In the beginning stages of method validation all analytes exhibited split peaks (Figure S 7), possibly due to partial blockage of the guard column frit. This recurring problem was resolved by reversing the flow through the column after each day of validation and after each sample batch to clear the initial blockage and to prevent future blockage (Mcnair & Polite, 2007). Furthermore,

during method validation a gradual increase in binary pump backpressure was observed after each injection. Since ammonium fluoride is only slightly soluble in methanol (National Centre for Biotechnology Information, 2023) it was assumed that this was caused by build-up of ammonium fluoride on the column during the time that the mobile phase was at 100% B. Surprisingly, retention time and peak shape were not affected by this. Further increase in pressure was minimised by adding a 10-minute time segment at the end of the method during which the mobile phase was 5% B (Figure S 8).

4.2 Derivatised method

4.2.1 Derivatisation

The derivatisation and LC-MS/MS method by (Nadarajah *et al.*, 2017) was developed and optimised for use on hair. For the derivatisation procedure used throughout method development, 2-HP was dissolved in methanol to a concentration of 5 mg/ml. 100 µl of derivatisation solution was added to standards and hair samples, which were incubated for 30 minutes at 60 °C (Nadarajah *et al.*, 2017). After incubation, 100 µl water was added so that the solution was similar to the starting mobile phase.

4.2.2 Mass spectrometry

For optimisation of MRM and source conditions 100 µl of derivatisation reagent was added to individual standards to a final concentration of 200 ppb and the mixture was incubated at 60 °C for 30 min. Formic acid was selected as the mobile phase modifier, because Nadarajah *et al.* (2017) showed that formic acid gave higher responses for cortisol and cortisone than ammonium hydroxide, while offering an 83-fold increase in DHEA response. Mobile phase A consisted of 0.2% formic acid in water and mobile phase B was methanol.

MRM conditions were first optimised by direct injection using the Agilent MassHunter Optimiser software as in Section 4.1.1.1. However, during development of the chromatographic method it was indicated that 2-HP produced excessive background leading to incorrect product ions being detected. As a result, optimal MRM conditions were determined manually by injecting individual standards onto the column using the preliminary gradient established in Section 4.2.3. SIMs of the precursor ions was done to determine the optimal fragmentor energies, starting with a wide range around the instrument default, then narrowing the range. The collision energies and product ions were similarly optimised by product ion scans starting with collision energies determined by Nadarajah *et al.* (2017) and scanning in a mass range to cover the predicted mass of the product ions (Nadarajah *et al.*, 2017). Precursor ion scans were then done to confirm that the product ions did indeed originate from the correct precursor ions. The optimal MRM conditions are summarised in Table 4-6.

The Agilent MassHunter Source and iFunnel Optimiser software was again used to optimise source conditions for each analyte. Because source optimisation through the column takes a significantly longer time than direct injection, fewer steps were tested for each parameter, especially for temperature-related parameters that require more time to stabilise between each value. The parameters as well as the increments at which they were tested can be viewed in Table S 3, while the optimised parameters are summarised in Table 4-7.

Next, the product ions of the stable isotopes were determined by a product ion scan using the same fragmentor voltages and collision energies that were optimised for their respective analytes. The MRM conditions of the stable isotopes were added to the method and are also shown in Table 4-6.

Table 4-6: The MRM conditions of the derivatised analytes and stable isotopes including the incorrect product ions identified due to excess 2-HP derivatisation reagent.

Compound	Molecular weight (g/mol)	Fragmentor energy (V)	Precursor Ion (m/z)	Collision energy (eV)	Product ion (m/z)	Incorrect product ion (m/z)
Cortisol-2HP	453.3	192	454.3	47	95.1	225.8
Cortisone-2HP	451.3	175	452.3	45	121	100.1
DHEA-2HP	379.3	172	380.3	47	95.1	270.8
Cortisol-d_4-2HP	457.3	192	458.3	47	121	n/a
Cortisone-d_8-2HP	459.3	175	460.3	45	125.1	n/a
DHEA-d_2-2HP	381.2	172	382.2	47	95.1	n/a

Table 4-7: The final source conditions optimised for the derivatised method.

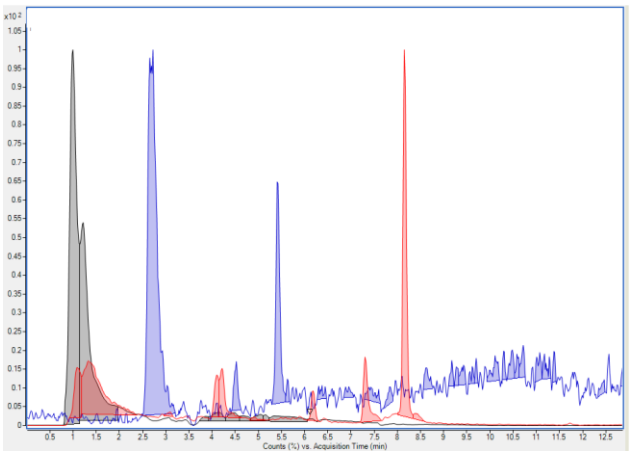
Capillary voltage (V)	Drying gas flow (L/min)	Drying gas temperature (˚C)	Nebulizer (psi)	Nozzle voltage (V)	Sheath gas flow (L/min)	Sheath gas temperature (˚C)
3000	4	150	20	0	12	400

4.2.3 Liquid chromatography

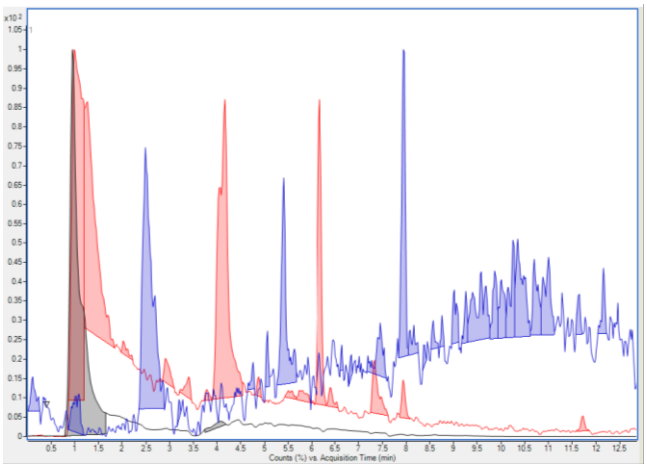
To establish the preliminary retention sequence of analytes, a 200 ppb standard mixture was injected onto the same column and using the same chromatographic method as initially used for the non-derivatised method. This was done because methods described for derivatised analytes

used flow rates that would produce backpressures that exceed those that the binary pump system could handle (Nadarajah *et al.*, 2017; Sosvorova *et al.*, 2015). Using the initial MRM conditions, multiple peaks were seen for all analytes, making determination of retention time impossible. Individual standards were then injected, but multiple peaks for all analytes were still present (Figure 4-7 a-c). The preliminary retention time was determined from peaks that were seen only in the chromatogram of the respective standard and not in others These retention times can be seen in Table S 4. The other peaks possibly resulted from excess background from the 2-HP reagent, which was confirmed by injection of 2-HP alone (Figure 4-7 d). The first 3 minutes in which the 2-HP eluted was set to go to waste so that excess 2-HP would not enter the MS and affect ionisation and detection. The time after which the analytes eluted was also set to waste to later prevent the entrance of unnecessary compounds that may be present in hair samples.

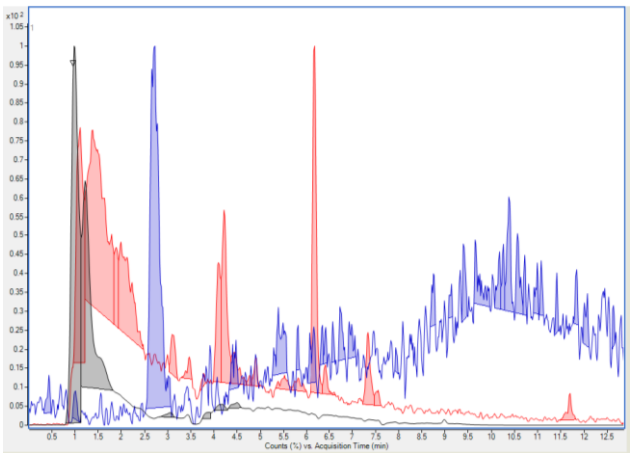
a) Cortisol



b) Cortisone



c) DHEA



d) 2-HP

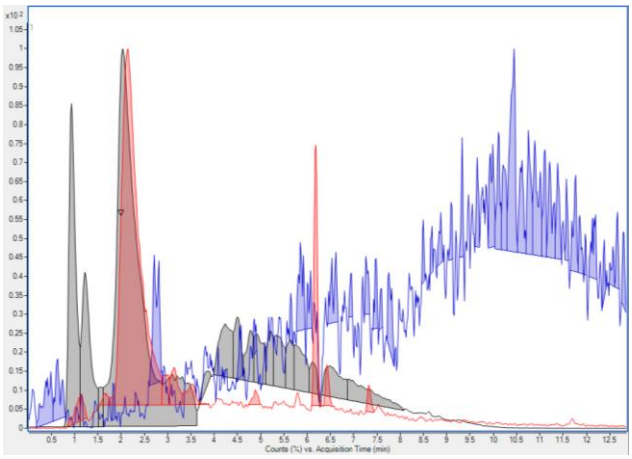


Figure 4-7: MRM chromatogram of each individual derivatised standard a) cortisol, b) cortisone, c) DHEA and d) 2-HP alone using the MRM transitions (red: cortisol, blue: cortisone, black: DHEA) determined by direct injection. Peaks were scaled to the most abundant peak in the chromatogram.

When establishing the suitability of the blank matrix more closely eluting peaks were observed that were not visible when higher concentrations of analytes were present. Thus, further optimisation of chromatographic separation was required. The separation was optimised using pooled blank hair matrix spiked with low analyte concentrations. The post time and time at 100% mobile phase B were again evaluated as described in Section 4.1.2. The post time and time at 100% mobile phase B were 3 and 5 minutes respectively. The final chromatographic method is summarised in Table 4-8, while the chromatographic profile and retention times can be seen in Figure 4-8 and Table 4-9, respectively.

Table 4-8: The liquid chromatographic mobile phase gradient. Indicating the times over which the percentage mobile phase B changes. The flow rate was 0.2 ml/min and the column temperature was 45° C.

Time (min)	% Mobile phase B
0	30
11	51
14	51
17	100
27	100
30	30

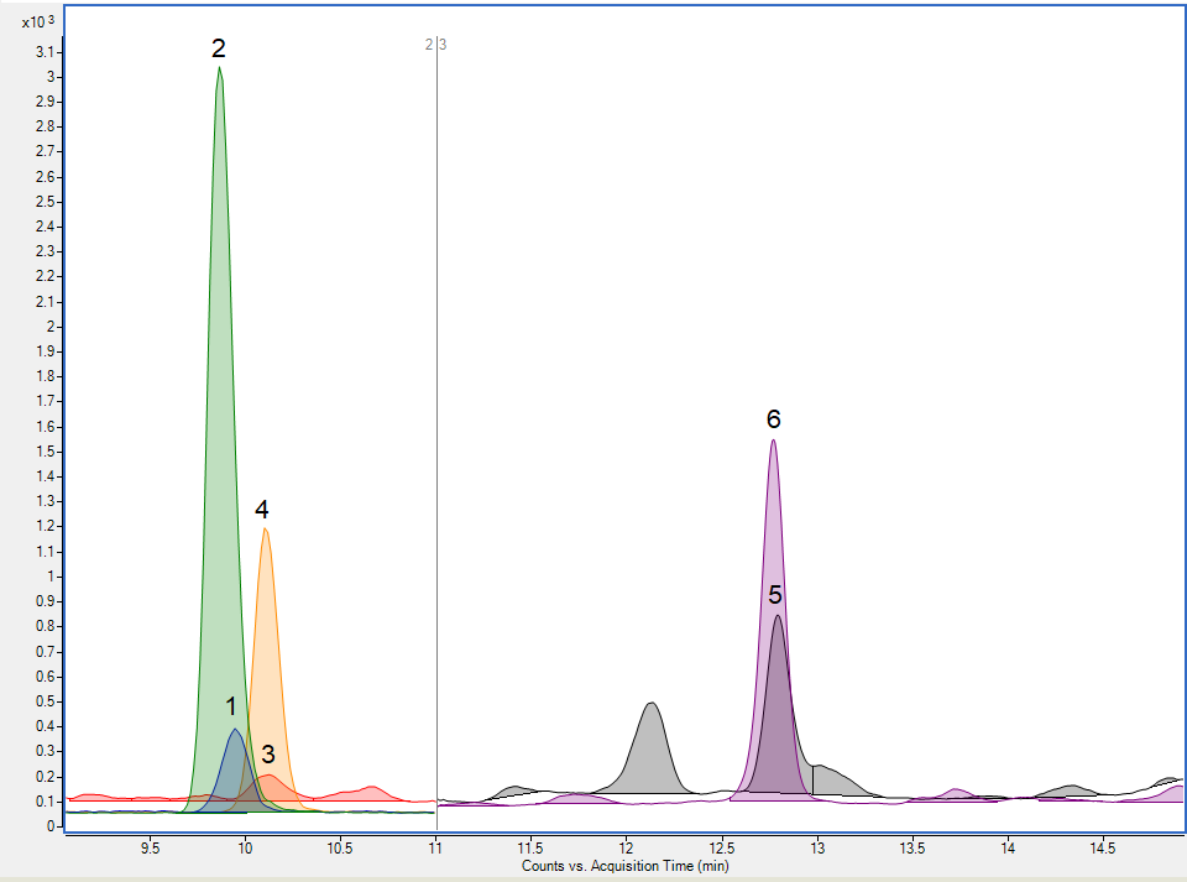


Figure 4-8: Chromatogram showing the separation of the derivatised analytes in a medium concentration spiked blank hair sample. 1. Cortisone-2-HP, 2. Cortisone-d_8-2HP, 3. Cortisol-2HP, 4. Cortisol-d_4-2HP, 5. DHEA-2HP, 6. DHEA-d_2-2HP.

Table 4-9: The retention times of each derivatised analyte for the final chromatographic method

Analyte	Retention time (min)
Cortisol-2HP	10.12
Cortisol-d_4-2HP	10.11
Cortisone-2HP	9.95
Cortiosone-d_8-2HP	9.87
DHEA-2HP	12.79
DHEA-d_2-H2P	12.78

4.2.4 Sample preparation and derivatisation

The sample preparation method developed in Section 4.1.3.1 was used and no further optimisation thereof was required. Improved responses of DHEA compared to the non-derivatised method meant that the carrier effect was no longer needed. Therefore, the concentration of DHEA-d_2 used for the remainder of derivatised-method development was 5 ppb (close to the upper expected physiological value), while the concentration of cortisol and cortisone stable isotope was kept the same as for the non-derivatised method.

The derivatisation method by Nadarajah *et al.* (2017) used 50 µl of 5 mg/ml 2-HP for saliva. To determine the method's applicability in hair, 2 different volumes (50 µl and 100 µl) were added to dried extracts of pooled hair samples from 6 individuals. From Figure 4-9 a) it is evident that 50 µl 2-HP was insufficient to completely react with all analytes. Therefore, greater volumes were then tested, which included 150 µl and 200 µl (Figure 4-9 b). The decrease in response with increasing 2-HP volume indicates that at 100 µl 2-HP is in excess and any volume greater than this would lead to ion suppression.

a) b)

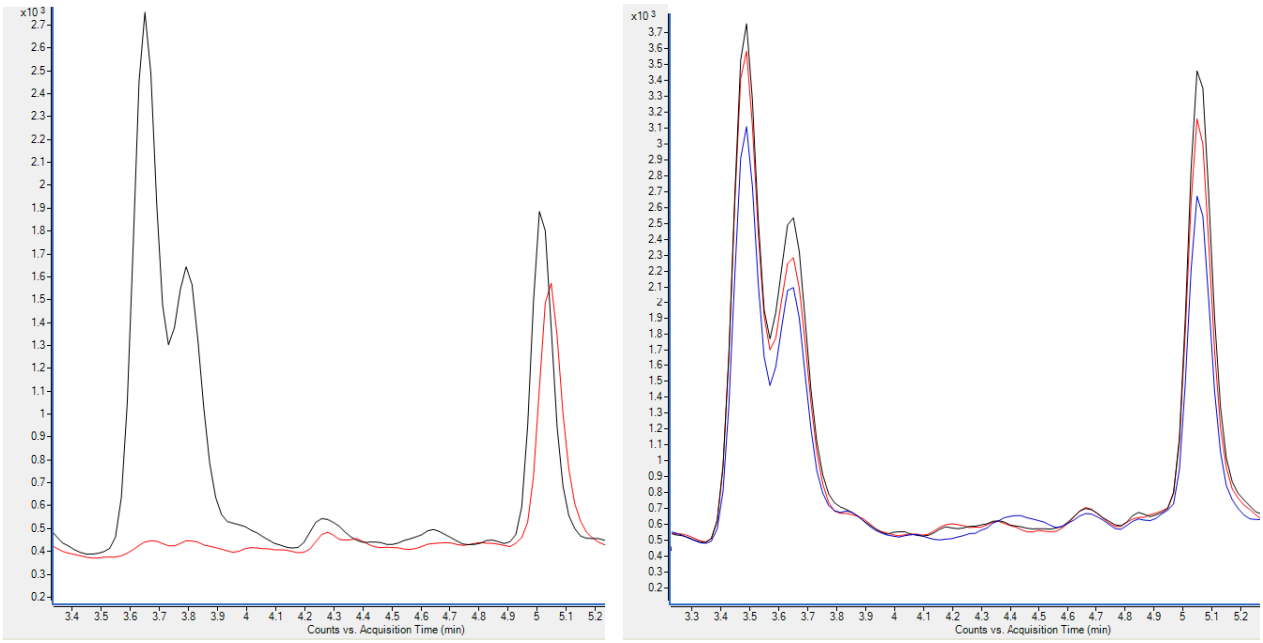


Figure 4-9: Total ion chromatograms showing how responses are affected by different 2-HP volumes. a) volumes tested were 50 µl (red) and 100 µl (blue). b) volumes tested were 100 µl (black), 150 µl (red) and 200 µl (blue).

4.2.5 Further optimisation

To determine the optimal injection volume 1 µl, 2 µl, 5 µl and 10 µl were tested. Figure 4-10 shows that responses increased with increasing injection volume across all volumes tested. However, peak broadening when injecting 5 µl or 10 µl indicated that the column was overloaded. Samples were then diluted to half the concentration and injection volumes were tested again. 5 µl was the best injection volume for the diluted sample. This volume was also compared to the injection volumes of the undiluted samples in Figure 4-10. Peak broadening was minimised and responses were enhanced above those of all undiluted injection volumes, revealing that aside from column overloading higher concentrations of other compounds in the undiluted sample may have led to ion suppression.

Lastly, a time segment was added at 11 minutes to scan for DHEA separately from cortisol and cortisone and the dwell times were optimised. The baseline was lowered for all analytes, and the optimal dwell times were 400 ms, 100 ms, and 200 ms for cortisol, cortisone and DHEA respectively.

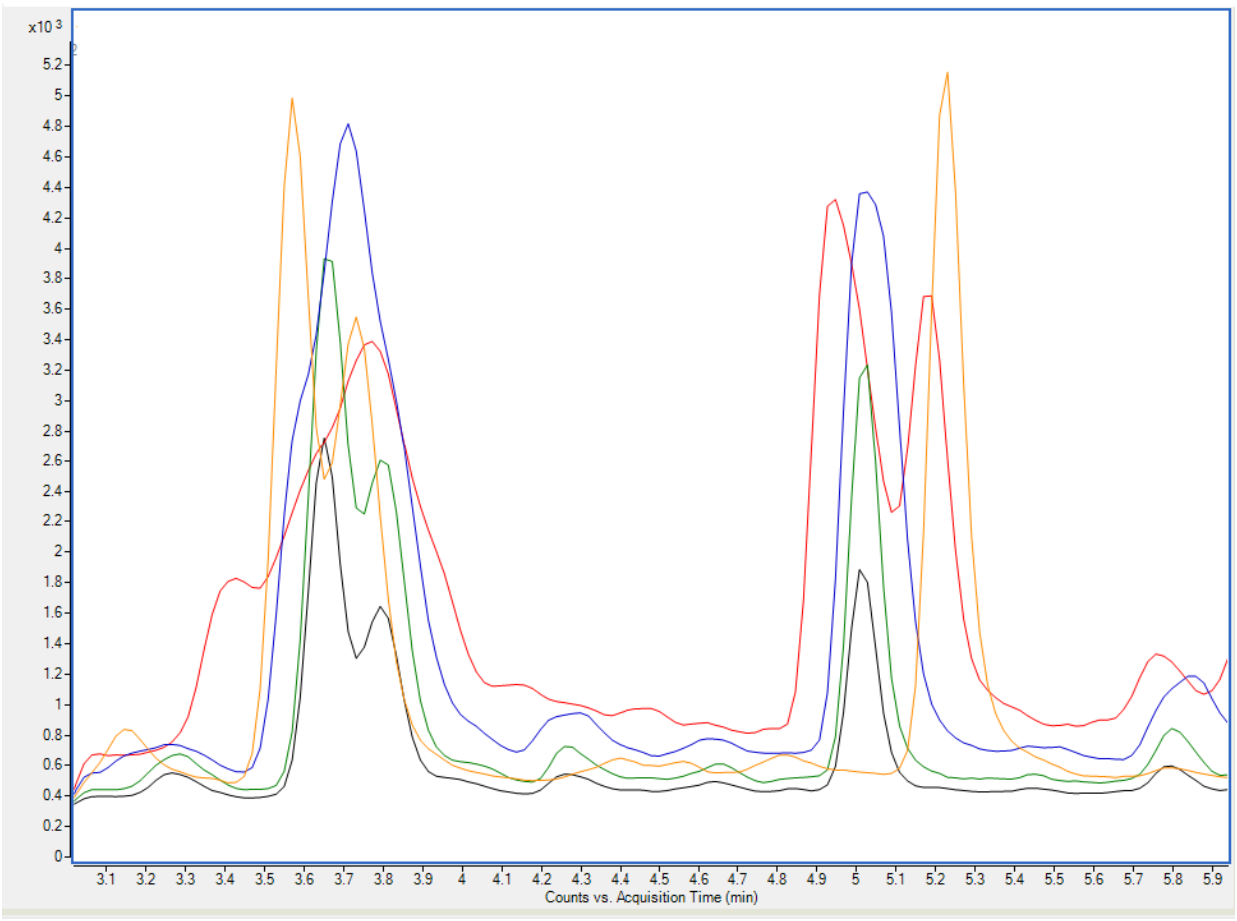


Figure 4-10: Total ion chromatogram showing the effect of injection volume in combination with sample dilution on analyte response. The volumes tested without dilution include 1 µl (black), 2 µl (green), 5 µl (blue) and 10 µl (red). The injection volume of the diluted sample was 5 µl (orange).

4.3 Comparison of derivatised and non-derivatised method

To determine which method would be best suited for the intended application, the selectivity and sensitivity of the derivatised and non-derivatised methods were compared. Chromatograms of the derivatised and non-derivatised methods obtained from medium concentration spiked blank matrix can be seen in Figure 4-11 a) and Figure 4-11 b), respectively. In the derivatised method there are peaks with the same MRM transitions that closely elute with cortisol, which could overlap at higher concentrations and may interfere with quantification. Although several peaks can be seen close to DHEA in the non-derivatised method, better separation could be achieved for both cortisol and DHEA.

a) b)

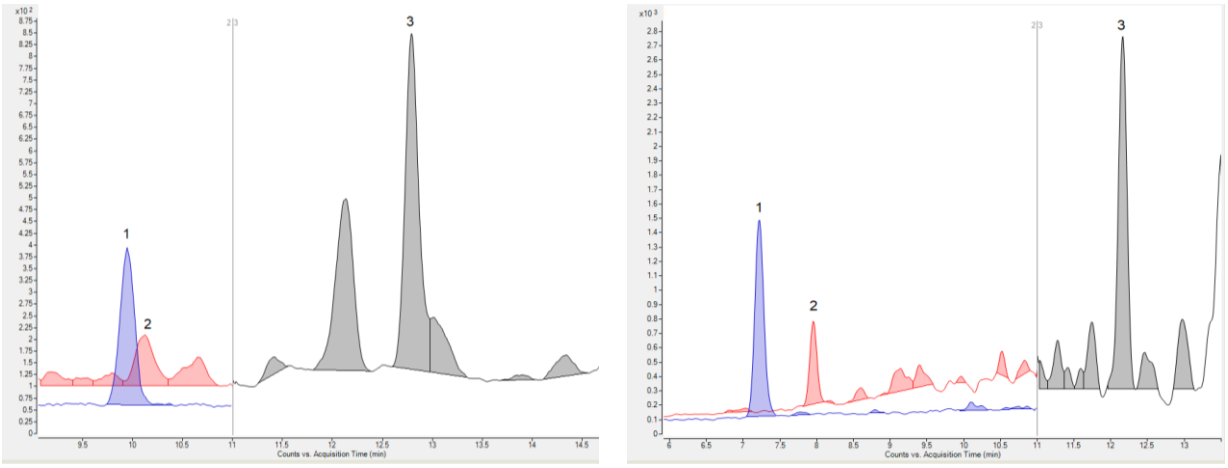


Figure 4-11: Chromatogram of the final a) derivatised and b) non-derivatised method obtained from blank matrix spiked with medium analyte concentrations. 1. Cortisone, 2. Cortisol, 3. DHEA.

The sensitivities of the methods were compared by calculating the LOD and LLOQ of each analyte from calibration curves (further explained in Section 5.1). From Table 4-10, it can be seen that both methods exhibited an LLOQ below the lowest expected physiological value for most analytes. Furthermore, the LLOQ for the non-derivatised method is lower than that of the derivatised method for cortisol and cortisone, but not for DHEA. Considering cortisol as the analyte of central importance to this study, the non-derivatised method was chosen for the remainder of the study due to its capability to quantify all analytes at the low concentrations expected in hair samples.

Table 4-10: The LLOQ of each analyte calculated for the derivatised and non-derivatised methods compared to the lowest expected physiological value. Values were obtained from spiked matrix calibration curves.

Analyte	LLOQ derivatised method (pg/mg)	LLOQ non-derivatised method (pg/mg)	Lowest physiological value (pg/mg)
Cortisol	3.12	0.93	1.38
Cortisone	1.60	1.26	6.85
DHEA	0.65	3.36	5.34

4.4 Conclusion

Although non-derivatised methods sensitive enough for hair cortisol and cortisone have been published (Gaudl *et al.*, 2016), the poor ionisation efficiency of DHEA explains the lack of such reports for this steroid hormone in hair. In this study the use of ammonium fluoride as mobile phase modifier to enhance analyte ionisation allowed for the development of a method that was sensitive enough for quantification of the low steroid levels expected in hair samples without the

need for derivatisation. This method could be further improved by considering post column infusion of ammonium fluoride to extend the column lifetime by eliminating build-up on the column (Schiffer *et al.*, 2022).

The derivatised method using 2-HP that was also developed in this chapter provided better sensitivity for DHEA than the non-derivatised method, but the sensitivity for cortisol was unacceptable for the purpose of this study. It follows that 2-HP is not an ideal derivatisation agent for simultaneous quantification of cortisol and DHEA and that other derivatisation methods should be considered where this is required. Possible improvements that can be made to the developed method is the consideration of SPE after derivatisation to get rid of excess 2-HP reagent so that greater volumes can be injected without ion suppression and peak broadening.

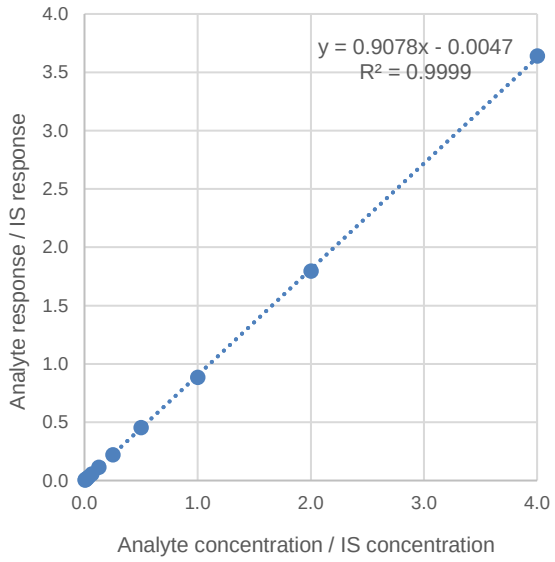
CHAPTER 5 METHOD VALIDATION

The non-derivatised method that was developed in Chapter 4 was validated to determine the suitability of the method for analytes in the hair matrix at the concentrations expected in samples. The validation parameters investigated included, linearity, sensitivity, accuracy, precision, carryover, selectivity, recovery, matrix effect, and stability after sample preparation. Unless otherwise stated validation was done in pooled blank matrix spiked with the specified concentrations and IS was added for quantification and to account for sample loss.

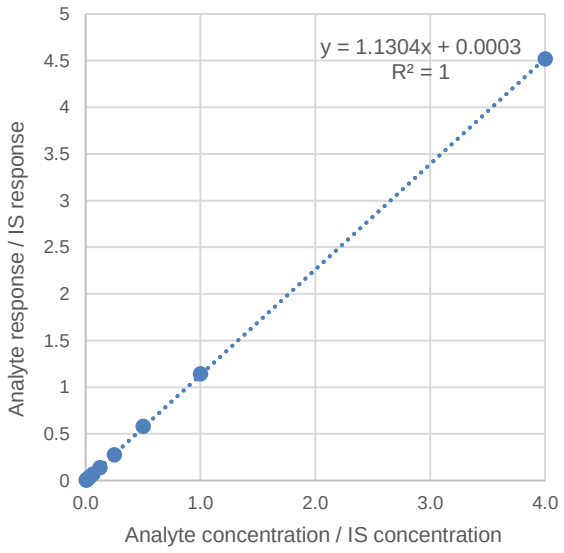
5.1 Linearity and sensitivity

The linearity is the degree to which the measurements obtained by the method are proportional to the concentration of the analytes within a defined range. A calibration curve was constructed from blank hair matrix spiked at 11 concentration levels covering the expected physiological range that were prepared in triplicate. The response of the analyte divided by the response of the respective ISs was plotted against the concentration of the analyte divided by the concentration of the IS (Honour, 2011; Wal *et al.*, 2010). The fit of the trendline was determined by least squares regression with a correlation coefficient (R^2) of >0.99 indicating linear responses (Honour, 2011). The concentration ranges that were covered as well the expected physiological range (Gao *et al.*, 2013; Raul *et al.*, 2004; Voegel *et al.*, 2020; Wosu *et al.*, 2015; Zhang *et al.*, 2018) are displayed in Table 5-1. The calibration curves for each analyte as well as the R^2 values and calibration curve equations can be seen in Figure 5-1. The method was linear for cortisol and cortisone, but when using DHEA-d_2 as the internal standard for quantifying DHEA the calibration curve was not linear (Figure 5-1c). The lack of linearity was possibly due to sufficient amounts of unlabelled analyte present in the stable isotope to contribute to the DHEA response (Garland & Barbalas, 1986). Cortisol-d_4 was then used for quantification instead, because Garland and Barbalas (1986) determined that when a high ratio of stable isotope to the analyte is used for a carrier effect, a separate compound should be used as an IS. From this a linear calibration curve could be constructed for DHEA (Figure 5-1d).

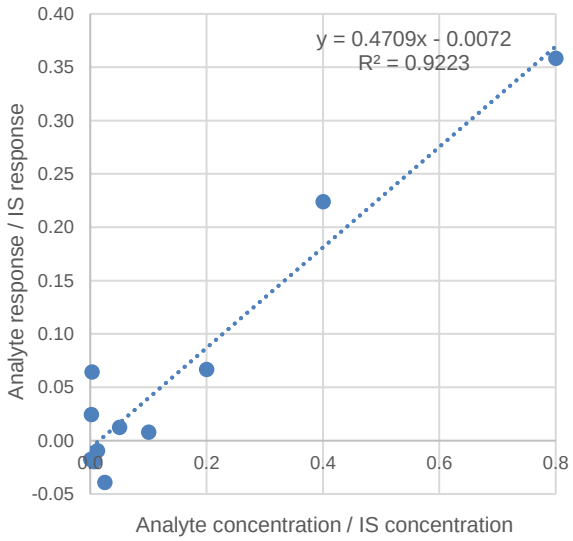
a) Cortisol



b) Cortisone



c) DHEA using DHEA-$d_2$as IS



d) DHEA using cortisol-d_2 as IS

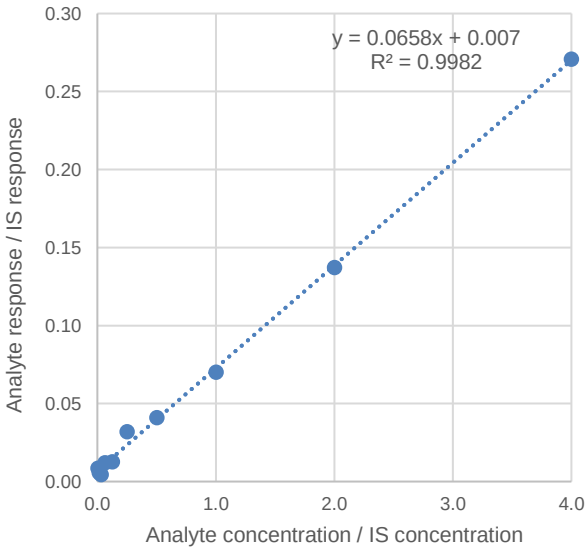


Figure 5-1: Calibration curves of each analyte indicating the linearity and the calibration equation.

Table 5-1: Table displaying the concentration range over which linearity was tested, the correlation coefficient for each analyte, and the physiological range from literature.

Analyte	Range evaluated (pg/mg)	Linear range (pg/mg)	R^2	Physiological range (pg/mg)
Cortisol	0.391 – 400	0.391 – 400	0.999	0.8 – 91.2
Cortisone	0.781 – 800	0.781 – 800	1	6.1 – 542.2
DHEA	0.391 – 400	0.391 – 400	0.998	1.9 – 58.3

The sensitivity of the method is described by the LOD and LLOQ, which were calculated by a calibration approach, according to Equation 1 and Equation 2. Five low concentrations resulting in a linear calibration curve were used (Kadian *et al.*, 2016; Shrivastava & Gupta, 2011). The upper limit of quantification (ULOQ) was taken as the highest concentration used in the calibration curve (Wal *et al.*, 2010). The linearity, LOD and LLOQ of the derivatised method were investigated in the same way, using the same concentration ranges, for method comparison in Section 4.3. The LOD and LLOQ of each analyte in the non-derivatised method are shown in Table 5-2 along with the concentration range that was used for the calculation. The LLOQ of cortisone and DHEA are below the lowest expected physiological concentration. Although the LLOQ of cortisol lies above the lowest expected physiological concentration, it is close to this value and the concentration in most samples will likely be above this. These results indicate that the method will likely be capable of accurately and precisely quantifying the analytes in biological samples.

Equation 1: The formula to calculate the limit of detection.

$$LOD = 3 \times \frac{standard\ deviation\ of\ y\ residuals}{slope\ of\ calibration\ curve}$$

Equation 2: The formula to calculate the lower limit of quantification.

$$LLOQ = 10 \times \frac{standard\ deviation\ of\ y\ residuals}{slope\ of\ calibration\ curve}$$

Table 5-2: Table displaying the LOD and LLOQ of each analyte as well as the concentration ranges used to calculate them.

Analyte	Range used to calculate LOD and LLOQ (pg/mg)	LOD (pg/mg)	LLOQ (pg/mg)
Cortisol	0.391 – 6.250	0.278	0.927
Cortisone	0.781 – 12.500	0.390	1.301
DHEA	1.56 – 100	1.008	3.359

5.2 Carryover

Carryover of the method was assessed to determine if there were any analytes that remained in the LC system, either in the needle or on the column after an injection that could possibly affect the following injection. This was done by comparing a blank sample injected before and a blank sample injected after an injection of a blank spiked with ULOQ concentration (Kadian *et al.*, 2016). No change in responses near analyte retention times was observed, indicating that there was no carryover.

5.3 Accuracy, precision, and dilution integrity

Inter-day and intraday accuracy and precision were tested in triplicate over three consecutive days at five concentration levels, namely LLOQ, LQC, MQC, HQC and ULOQ. Together with the QC samples, the accuracy and precision of dilution integrity samples were investigated to determine whether samples that had high analyte concentrations could be diluted and still be accurately and precisely quantified. These samples consisted of double the concentration of ULOQ samples that were diluted either two or four times (Wal *et al.*, 2010). Precision was calculated as the relative standard deviation (RSD) between measurements. The acceptability criteria were RSD ≤ 20% at LLOQ and < 15% at all other concentrations. To determine accuracy, the calculated concentration was expressed as a percentage of the known concentration which was considered acceptable when within 20% at LLOQ or within 15% at higher QC concentrations (González *et al.*, 2014; Kadian *et al.*, 2016). Both intra- and inter-day precision and accuracy were acceptable for all concentrations, as well as the dilution integrity samples of cortisol and cortisone (Table 5-3Table 5-4). The accuracy and precision of the method for DHEA only became acceptable at the ULOQ and for dilution integrity samples. Since these concentrations were far greater than the expected physiological concentration, DHEA was excluded from all further analysis. The cross contribution of high DHEA-d_2 concentrations to DHEA response could be the cause of impression and inaccuracy (Garland & Barbalas, 1986), even when cortisol-d_4 was used as the IS for DHEA quantification.

Table 5-3: Intraday accuracy and precision.

Concentration level	Cortisol		Cortisone		DHEA	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
LLOQ	93.21	13.22	82.17	9.03	696.68	57.15
LQC	94.81	1.93	92.68	3.24	455.54	57.88
MQC	98.74	4.59	97.74	3.54	349.01	14.35
HQC	100.02	1.28	99.21	1.19	7.94	11.38
ULOQ	99.58	2.85	96.81	2.06	15.09	4.09
Diluted x2	101.67	0.06	93.46	0.73	98.58	1.09
Diluted x4	100.11	4.65	93.45	4.40	84.01	2.17

Table 5-4: Inter-day accuracy and precision.

Concentration level	cortisol		Cortisone		DHEA	
	accuracy (%)	RSD (%)	accuracy (%)	RSD (%)	accuracy (%)	RSD (%)
LLOQ	83.68	10.53	80.10	14.49	-10747.00	119.34
LQC	99.99	4.72	93.60	4.64	-1914.04	119.99
MQC	101.31	3.59	98.31	1.48	-503.61	76.03
HQC	101.99	1.94	103.47	5.42	-203.80	77.93
ULOQ	101.39	1.58	97.81	3.46	35.87	12.36
Diluted x2	100.72	3.80	92.57	3.46	95.37	3.89
Diluted x4	102.67	2.20	95.07	1.63	86.41	4.65

5.4 Selectivity

The selectivity of the method is the ability to distinguish between the analyte and any other endogenous compound in the sample. To assess the selectivity of the method, a blank sample was compared to an LLOQ sample. Chromatograms of the results are shown in Figure 5-2. For each analyte there is a peak in the blank sample at the exact analyte retention time that has a response of approximately 50% of that of the LLOQ. Rather than indicating interference, these peaks show that not all analytes were completely washed out in the sections of hair that were used to prepare the blanks, suggesting that longer hair sections should be used instead as was done by (Gao *et al.*, 2013). There was another peak in the blank sample that closely eluted with cortisone. However, the response of this peak was lower than 20% of the response of the LLOQ and was thus not considered as an interference (Wal *et al.*, 2010). There were peaks either at the same retention time (cortisone-d_8) or close to the retention time (cortisol-d_4 and DHEA-d_2) of all stable isotopes, but responses were less than 5% of responses in the sample to which stable isotopes were added, indicating once again that these peaks would not interfere with analyte quantification (Wal *et al.*, 2010). To confirm whether the peaks at the same retention time as analytes were indeed residual amounts of analytes in blanks rather than other compounds, a second specific product ion (qualifier ion) could be added to the method for more selective analyte identification (Honour, 2011). Furthermore, samples could be spiked with known potential interfering compounds to confirm that they would not interfere with analyte quantification (González *et al.*, 2014).

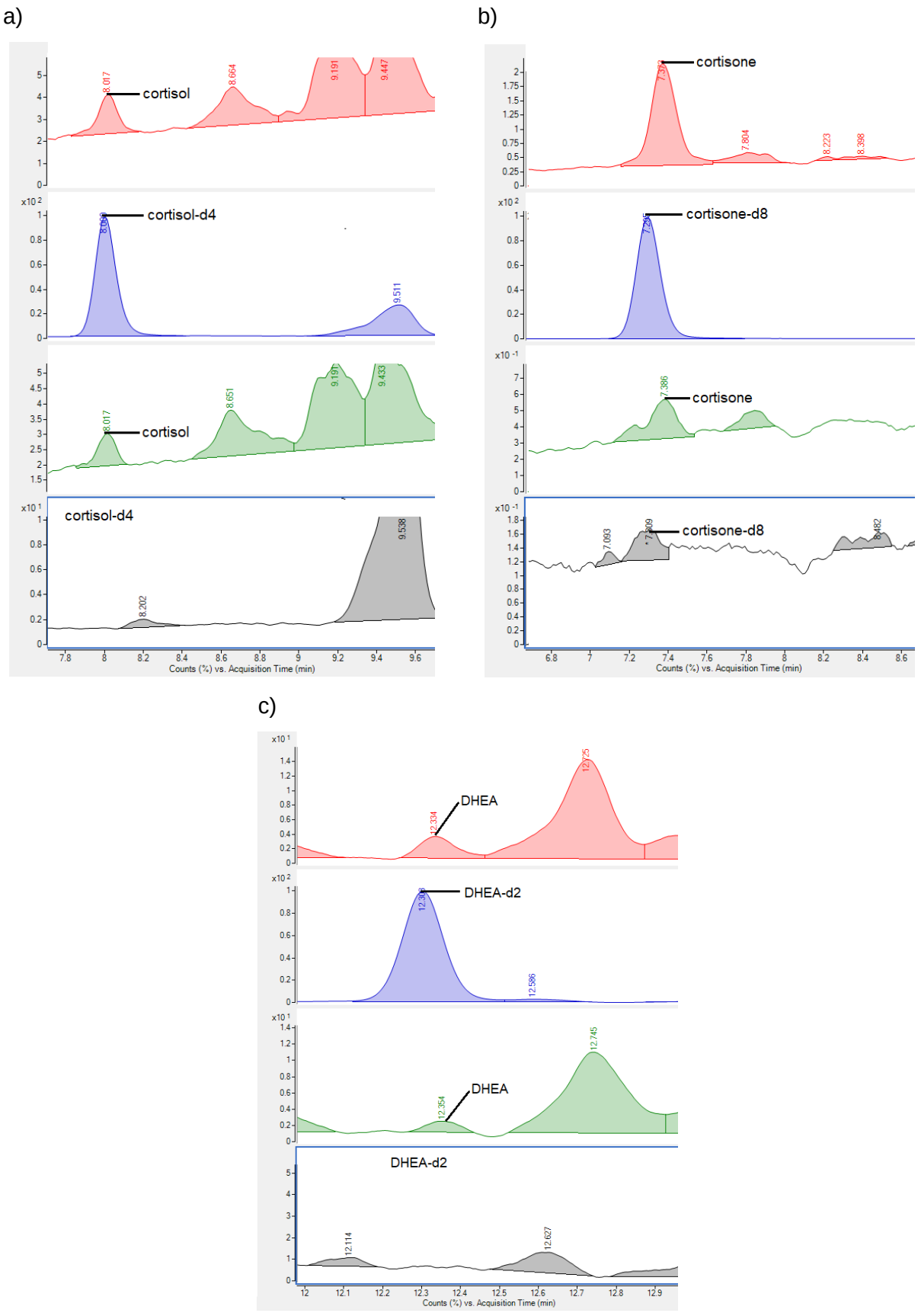


Figure 5-2: MRM chromatograms of analytes and isotopes in a blank sample containing IS spiked with LLOQ compared to an un-spiked blank sample without IS. a) Cortisol, b) Cortisone, c) DHEA. Red: analyte in spiked sample, Blue: isotope in spiked sample, Green: analyte in blank. Black: isotope in blank.

5.5 Recovery

The recovery of the method for LQC, MQC and HQC was tested in triplicate by comparing responses of samples spiked before and after SPE. The results displayed in Table 5-5, show that the recoveries of cortisol and cortisone were close to 100% and were consistent across the different QC concentrations (González *et al.*, 2014; Wal *et al.*, 2010). This demonstrates that sample preparation is repeatable and that very little analyte is lost in the process.

Table 5-5: The recovery of analytes at different concentrations.

Concentration level	Recovery (%)	
	Cortisol	Cortisone
LQC	99.22	97.69
MQC	94.50	95.75
ULOQ	96.01	97.17

5.6 Matrix effect

The matrix effect is when components in the matrix have an impact on ionisation and consequently quantification of the analytes. The degree to which this occurs is described by the matrix factor (MF), which is expressed as the response in the presence of matrix components relative to the response with no matrix components present. In the absence of matrix effects the MF will be 1, while ion suppression is indicated by a an MF below 1 and ion enhancement by an MF above 1 (González *et al.*, 2014).

The matrix effect was determined according to Equation 3 using blank hair samples from 6 individuals spiked at LLOQ concentration after SPE (Binz *et al.*, 2016; Wal *et al.*, 2010). MFs of 0.80 for cortisol and 0.89 for cortisone indicate that there is ion suppression for both analytes.

Equation 3: The formula to calculate the matrix factor.

$$MF = \frac{response\ in\ spiked\ matrix}{response\ in\ pure\ standards}$$

5.7 Stability

In certain circumstances in a laboratory, it might not be possible to analyse samples immediately or samples might need to be reanalysed after some time. The storage conditions of samples during these instances need to be assessed to determine if results from these samples are reliable. The stability of analytes in prepared samples were tested in triplicate under different

storage conditions. These included 4 days in the freezer (-20°), refrigerated (4°C) for 4 days and in the autosampler (25°C) for 24 hours. The stability results displayed in Table 5-6 are the responses of samples after storage expressed as a percentage of the response from fresh samples (Honour, 2011). Except at the LLOQ stability was close to 100% for all storage conditions. Nevertheless, refrigeration gave the most consistent results across the different concentrations tested.

Table 5-6: The stability of analytes at different concentrations under different storage conditions.

Concentration level	Cortisol stability (%)			Cortisone stability (%)		
	-20°C (4 days)	4°C (4 days)	Autosampler (24 hours)	-20°C (4 days)	4°C (4 days)	Autosampler (24 hours)
LLOQ	69.74	78.52	99.11	115.64	110.12	83.07
LQC	90.82	96.61	93.76	106.53	98.36	94.66
MQC	104.73	99.47	92.72	101.48	95.64	98.28
HQC	99.40	98.61	99.76	90.87	97.87	110.23
ULOQ	98.31	98.64	102.66	92.41	96.70	108.34

5.8 Conclusion

The validation results discussed throughout this chapter show that the method is selective, sensitive, accurate and repeatable for cortisol and cortisone in hair over the expected concentration range. Although the method appears to be linear for DHEA in hair matrix it is, unfortunately neither accurate nor repeatable and this analyte had to be excluded. The inaccurate and imprecise results may have been caused by large DHEA isotope concentrations used in an attempt to improve the sensitivity by the carrier effect, showing that the concentration of stable isotopes should be carefully considered and optimised for the development of a sensitive method that is also accurate and repeatable.

CHAPTER 6 METHOD APPLICATION

6.1 Introduction

Several studies investigating the effects of COC use on cortisol levels and chronic stress report conflicting results. To gain clarity on these contradictions, an in-depth assessment of several chronic stress markers in hair, including cortisol, cortisone, total glucocorticoids and cortisol/cortisone ratios was done. The method that was developed and validated in the previous two chapters were used to measure hair cortisol and cortisone. Furthermore, ethnicity was assessed as a covariate for these hair markers to determine if it needs to be accounted for in future studies.

6.2 Samples and LC-MS/MS analysis

Samples were collected as described in Section 3.4 **Error! Reference source not found.**. The sample groups were Caucasian users (n=24), Caucasian non-users (n=24), and Black African non-users (n=20). There was an insufficient number of Black African women using EE/DRSP to be included in the study. Most Black African women that used COCs reported using other hormonal compositions. Samples were randomised and analysed in three batches according to the developed method which is summarised in Section 3.5**Error! Reference source not found.**. Low, medium and high QC samples were also analysed in each batch. Cortisol and cortisone were quantified using their respective stable isotope internal standards according to the equations derived from the calibration curves. From these concentrations the following were calculated: cortisol/cortisone ratio and total glucocorticoid concentration (the sum of cortisol and cortisone). A summary of the calculated concentrations, sums and ratios from all participants (excluding values below the LLOQ) as well as the expected values obtained from literature (Chen *et al.*, 2019; Gao *et al.*, 2013; Quinete *et al.*, 2015; Raul *et al.*, 2004; Voegel *et al.*, 2020; Wosu *et al.*, 2015; Zhang *et al.*, 2018) can be seen in Table S 5. Three cortisol and four cortisone values were below the LLOQ and only one cortisone value was below the LOD. All other values were between the LLOQ and ULOQ. However, the lowest concentrations and medians of cortisol, cortisone and total glucocorticoids were lower than those in the literature. Although the median was higher than expected, the cortisol/cortisone was within the expected range.

6.3 Statistical analysis

Data pre-processing and multivariate statistics were carried out in MetaboAnalyst 5.0, while univariate statistics was done using GraphPad Prism 10.

6.3.1 Data preprocessing

The first step of data analysis was investigation of the QC samples to ensure that there was no variation in analyte measurements that would lead to unreliable results. Coefficients of variation (CV) for all analytes were below 15% and were considered acceptable.

Before data can be statistically analysed it needs to be normalised, transformed and scaled. Normalisation is usually required to account for differences in sample dilution such as seen in urine. The reported concentrations were already normalised by hair mass and no other normalisation technique was necessary. Data transformation reduces the skewness of the distribution, making it more normally distributed (Gaussian) in order to meet the assumptions that many statistical tests, are based on. Scaling serves to make different analytes comparable regardless of large concentration differences (Van den Berg *et al.*, 2006; Xia & Wishart, 2016). Different combinations of the transformation and scaling techniques available in MetaboAnalyst were tested. Log transformation and Pareto scaling resulted in the best distribution. The improvement in data distribution in both analyte and sample view are shown in Figure 6-1 and Figure 6-2.

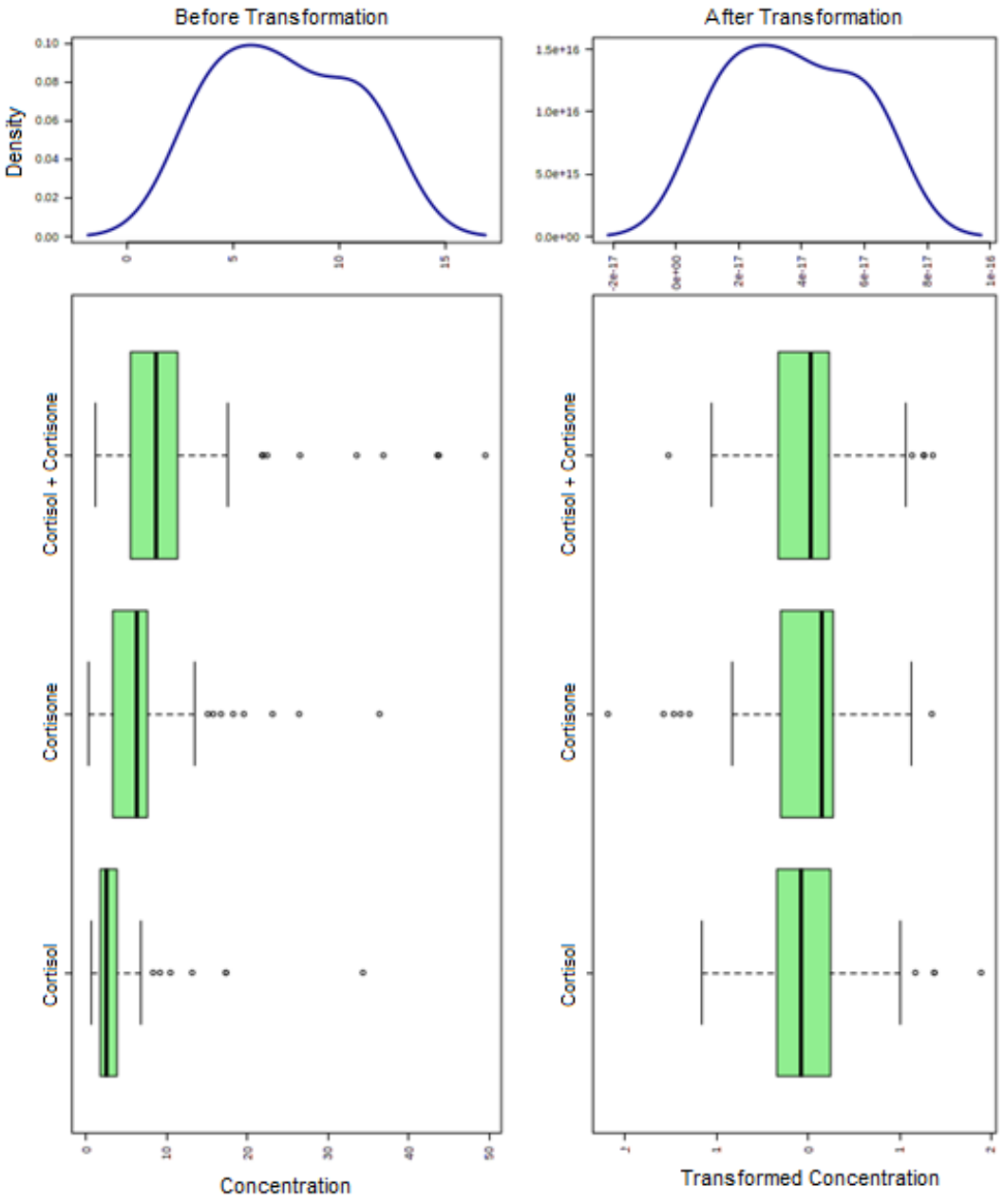


Figure 6-1: The distribution of the data in analyte view before (left) and after (right) normalisation and scaling.

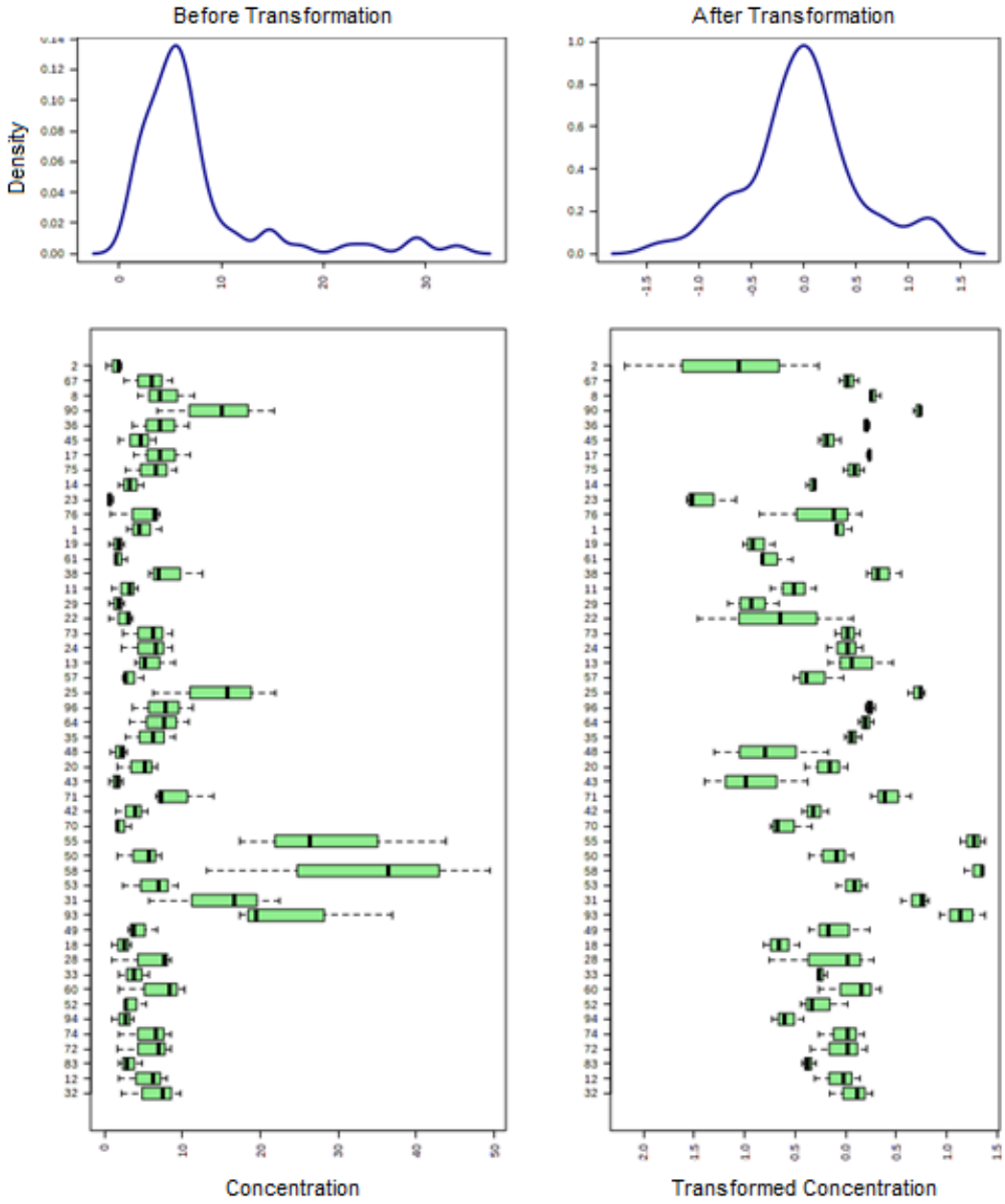


Figure 6-2: The distribution of the data in sample view before (left) and after (right) normalisation and scaling.

When analysing samples in multiple batches small differences in sample handling or environment may lead to systematic errors and variation in measurements between batches. The presence of a batch effect was investigated analysing cortisol, cortisone, and total glucocorticoids simultaneously, thus multivariate analysis. This was done by principal component analysis (PCA), which plots linear combinations of variables as principal components to maximally describe the variation with fewer dimensions (Ringnér, 2008). In the two-dimensional scores plot in Figure 6-3, a lack of clustering indicates that there wasn't a batch effect and that no batch adjustment was required before further analysis.

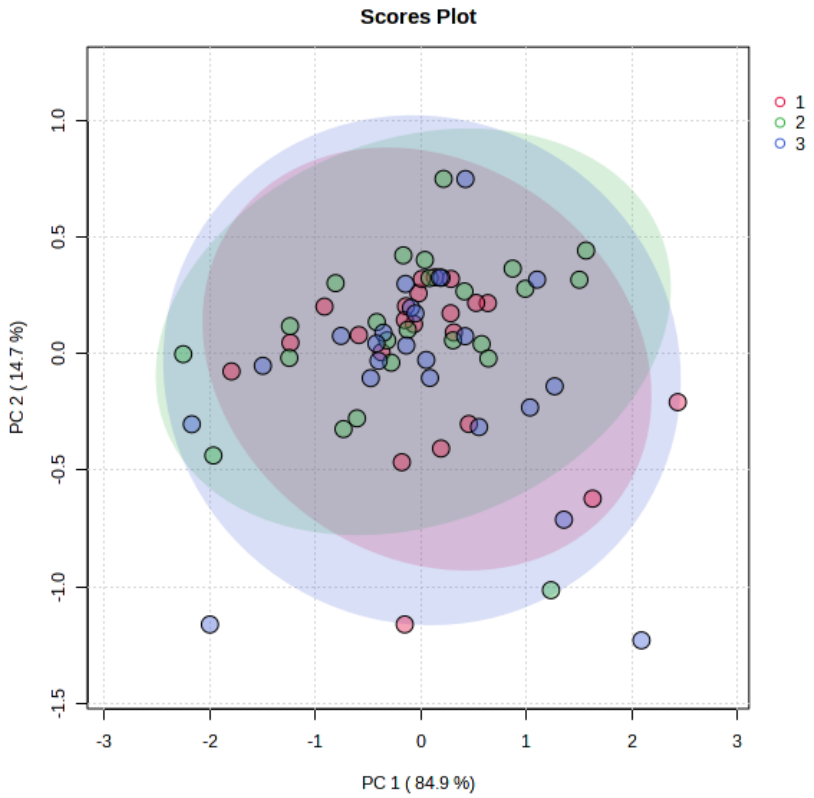


Figure 6-3: The two-dimensional scores plot of the PCA done to investigate the presence of a batch effect across the three batches that samples were analysed in. Each batch is represented by a different colour.

Outliers were detected next. Although only univariate statistics were used in this study, a multivariate approach to outlier detection was followed because the relationship between analytes was important. If a univariate approach to outlier detection was used, a sample that was an outlier for cortisol or cortisone might not have been an outlier for total glucocorticoids or the cortisol/cortisone ratio, and thus removing them would result in the unnecessary loss of meaningful data. PCA of cortisol, cortisone, and total glucocorticoids was again used to achieve this. The cortisol/cortisone ratio was not included because PCA inherently describes the relationship between variables (Ringnér, 2008). Visual inspection of the two-dimensional scores plot in Figure 6-4 revealed samples 80 and 93 as outliers. Nevertheless, only sample 80 was far enough away from the rest of the data points in the sample group to be considered as an extreme outlier and was removed from the data set.

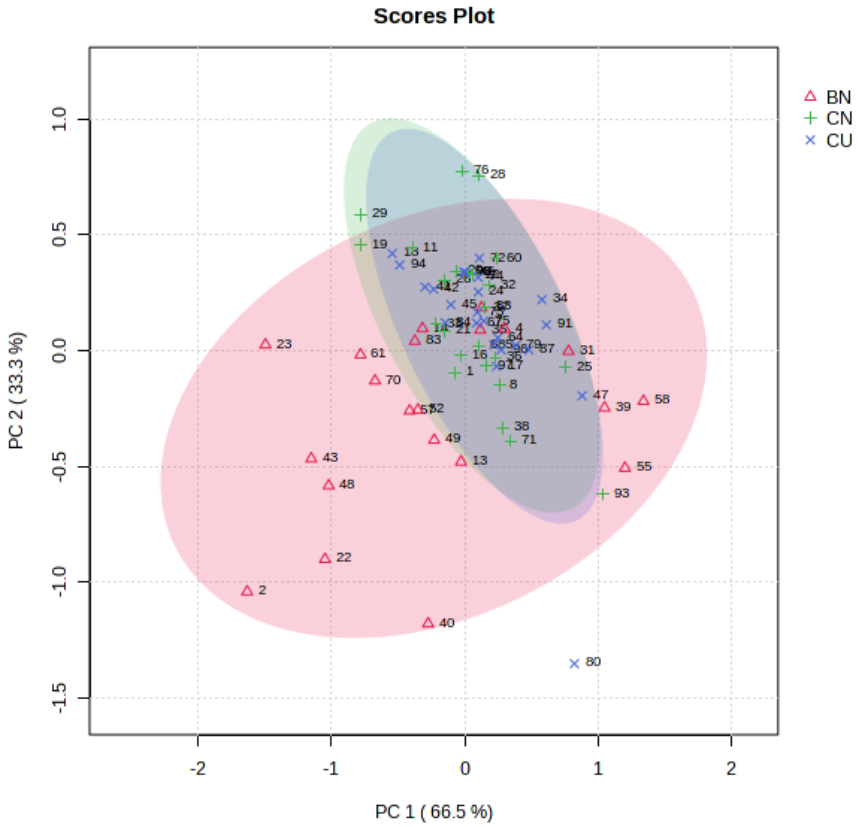


Figure 6-4: The two-dimensional scores plot of the PCA done to detect outliers in each sample group. Red: Black African non-users, green: Caucasian non-users, blue: Caucasian users.

Finally, covariates of cortisol and cortisone were assessed by calculating Pearson r correlation coefficients. Values between 0.5 and 1 indicate a strong positive correlation, while values between -1 and -0.5 indicate a strong negative correlation. Factors that were tested as possible covariates for cortisol and cortisone were the season of sample collection (summer or winter), ethnicity, BMI, participant age, sample storage time (months), and hair treatment. Hair treatments included heat treatment, chemical treatment (dye or relaxer), supplementation (hair food, coconut oil, or olive oil), and no treatment. None of the investigated parameters were correlated with cortisol or cortisone.

6.3.2 Multivariate statistics

Multivariate statistics are useful when many variables are analysed and the relationships between some of them may be important. In this study univariate statistics would be more beneficial due to the small number variables and the known relationships between cortisol and cortisone that can be studied more directly. Therefore, PCA was only used for visualisation of the sample groups. The PCA scores plot of cortisol, cortisone and total glucocorticoid after outlier removal can be seen in Figure 6-5. There are no clearly separated clusters between groups. However, samples of Caucasian users are more tightly clustered than both non-user groups, yet samples of Caucasian non-users are more tightly clustered those of Black African

non-users. This indicated that Black African non-users had the most variation in glucocorticoid levels while Caucasian users had the least variation.

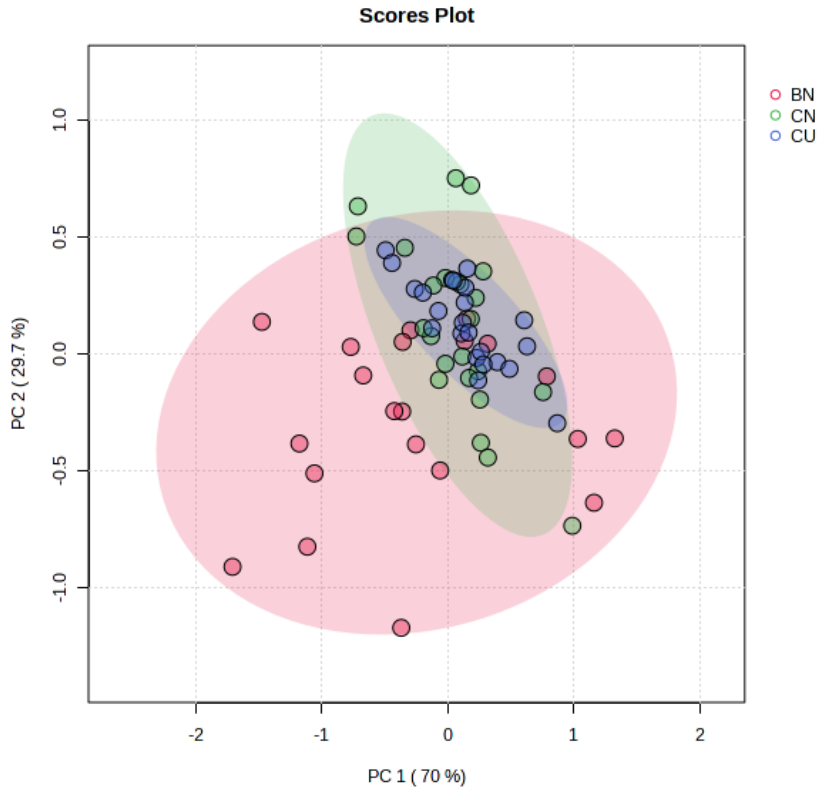


Figure 6-5: The two-dimensional PCA scores plot of each sample group after the outlier was removed. Red: Black African non-users, green: Caucasian non-users, blue: Caucasian users.

6.3.3 Univariate statistics

During univariate analysis one variable is analysed at a time. By determining how each variable, including glucocorticoid concentrations and their ratio, differs between sample groups, more insight can be gained on individual hormones and what their associations with each other mean.

Analysis of variance (ANOVA) is commonly used to determine whether or not a difference exists between the means of three or more sample groups. If the ANOVA results are positive, *post hoc* tests are done to distinguish between which groups there are statistically significant differences. There are many different *post hoc* tests that can be used and the choice should be carefully considered based on the nature of the data as well as the question to be answered. The Dunnett's test can be used with unequal sample sizes and allows for the comparison of selected groups to a control group. This minimises the number of tests for which the *p*-value needs to be controlled, which will be discussed shortly (McHugh, 2011; Sauder & DeMars, 2019). When groups have unequal variances, as is the case with the data in this study, the Dunnett's T3 version should be used (Lee & Lee, 2018; Sauder & DeMars, 2019).

Although the Dunnett's tests is usually applied as a post hoc test only after results of ANOVA are significant, Hothorn (2016) suggests the use of the Dunnett's test without prior ANOVA to avoid type II errors (false negatives). When multiple tests are performed on a data set, i.e., when comparing different groups and multiple variables, type I errors (false positives) can arise. To avoid this the *p*-value is adjusted by multiple comparisons tests such as Bonferroni or the false discovery rate (Lee & Lee, 2018). The *p*-value indicates whether or not there is a difference between groups, but the effect size must also be calculated to give an indication of the magnitude of the difference. The effect size takes sampling variation into account and allows for comparisons between different studies, regardless of sample sizes (Sullivan & Feinn, 2012).

Univariate analysis was done using GraphPad Prism 10. Data was log transformed to obtain a more normal distribution, but scaling was not applied because different analytes did not need to be comparable. Figure 6-6 shows the distribution of analytes before and after transformation. A Dunnett's T3 test was done on all variables. Caucasian non-users were selected as the group that Caucasian users and black African non-users were compared to. Multiple comparisons were corrected for by statistical hypothesis testing. A *p*-value ≤0.05 was considered significant. Effect size was calculated by the Cohen's *d* value, where 0.2 is a small effect, 0.5 is a medium effect, 0.8 is a large effect, and 1.3 is a very large effect (Sullivan & Feinn, 2012).

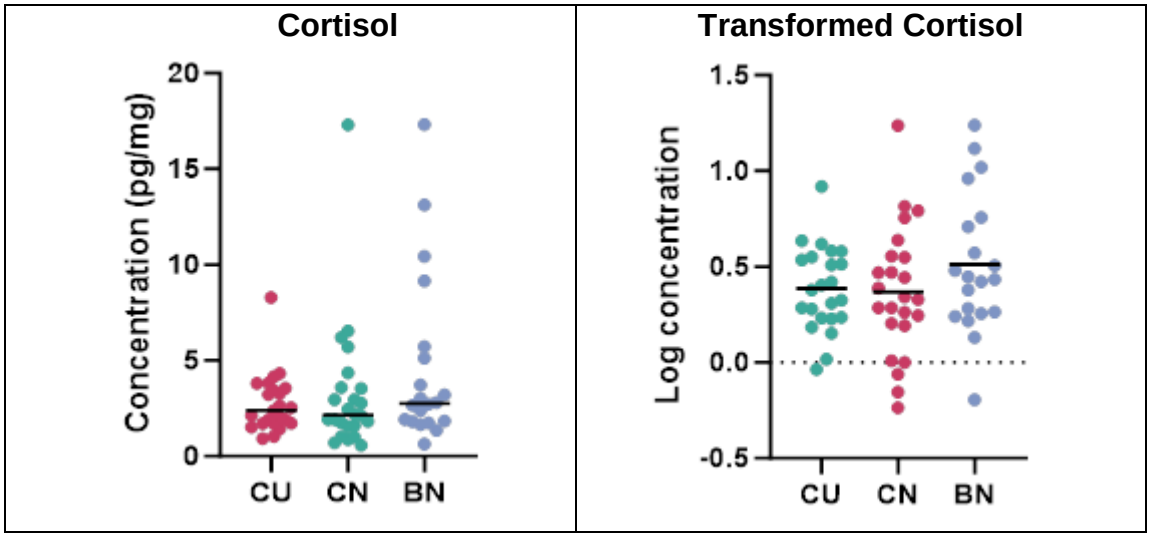


Figure 6-6: The distribution of data points for each variable before (left) and after (right) log transformation. CU: Caucasian users, CN: Caucasian non-users, BN: Black African non-users. The bars represent the medians of untransformed data and the means of transformed data. (Continues on next page)

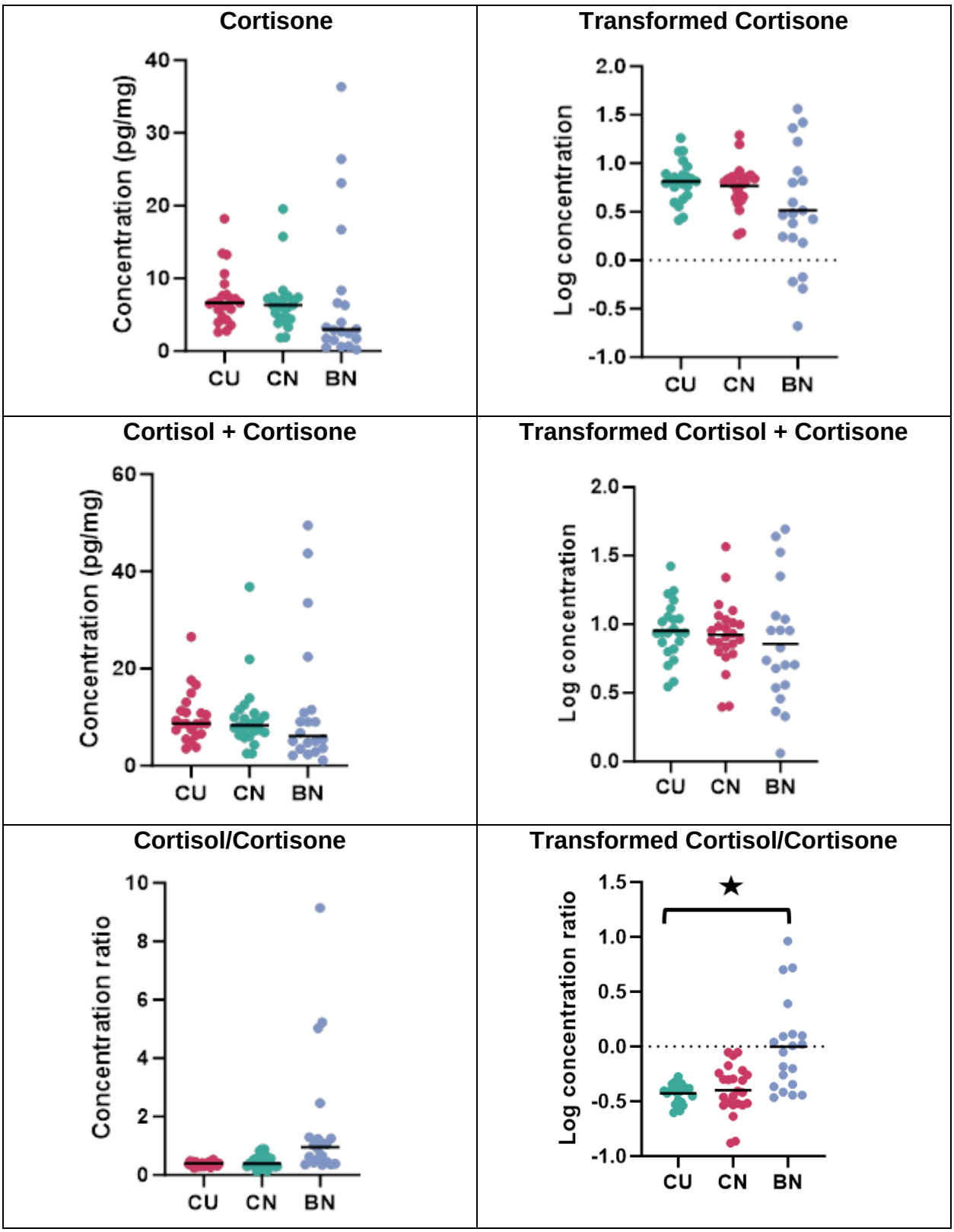


Figure 6-6 continued: The distribution of data points for each variable before (left) and after (right) log transformation. CU: Caucasian users, CN: Caucasian non-users, BN: Black African non-users. The bars represent the medians of untransformed data and the means of transformed data. Statistical differences between groups are indicated by stars.

The results of the Dunnett's T3 test, with adjusted and unadjusted *p*-values as well as effect sizes (calculated by Cohen's *d* value) are displayed in Table 6-1, while the transformed means and standard deviations (SD) of all variables in each group can be seen in Table 6-2. The descriptive statistics of the untransformed data of each group is summarised in Table S 6.**Error! Reference source not found.** The only variable that was statistically significant was the higher cortisol/cortisone ratio in Black Africans compared to Caucasians. This difference also had a large effect size. This was the result of slightly higher cortisol and slightly lower cortisone levels in Black Africans compared to the Caucasian groups. Although these differences were not statistically significant, they had small and medium effect sizes, respectively. For most variables the standard deviations of Caucasian users were comparable to those of Caucasian non-users, but the standard deviation for most metabolites of Black African non-users was greater than those of the other sample groups, indicating greater variation in this sample group. Body fat percentage had a large relative standard deviation within the Black African sample group (36.4%) compared to that of the Caucasian sample group (19.9%). Therefore, body fat percentage was assessed as a covariate of all variables within the Black African sample group to determine if it accounted for the variation within the group. All Pearson r correlation coefficients were between 0.10 and 0.15 indicating no correlation. Because the duration of COC use varied between a wide range (3 months to 8 years), its association with the stress markers in COC users was also investigated. No correlation was found, with Pearson r correlation coefficients between -0.18 and 0.12.

Table 6-1: The *p*-values from the Dunnett's T3 test with and without multiple testing adjustments, comparing COC-users (CU) to non-users (CN) and Black Africans (BN) to Caucasians (CN). The Cohen's *d* value indicates the effect size. Cohen's *d* value >0.2: small effect, >0.5: medium effect, >0.8: large effect, >1.3: very large effect.

Variable	CU vs. CN			BN vs. CN		
	Unadjusted *p*-value	Adjusted *p*-value	Cohen's *d*	Unadjusted *p*-value	Adjusted *p*-value	Cohen's *d*
Cortisol	0.830	>0.9999	0.04	0.182	0.780	0.38
Cortisone	0.455	0.991	0.23	0.089	0.493	0.57
Total glucocorticoids	0.649	1.000	0.13	0.563	0.998	0.18
Cortisol/cortisone	0.542	0.997	0.18	0.001	0.005	1.08

Table 6-2: The mean and standard deviation of each sample group (CU: Caucasian COC users, CN: Caucasian non-users, BN: Black African non-users) after log transformation.

Variable	CU		CN		BN	
	Mean	SD	Mean	SD	Mean	SD
Cortisol (pg/mg)	0.39	0.22	0.38	0.34	0.51	0.36
Cortisone (pg/mg)	0.81	0.21	0.76	0.23	0.51	0.6
Total glucocorticoids (pg/mg)	0.95	0.21	0.92	0.25	0.86	0.44
Cortisol/cortisone	-0.43	0.09	-0.4	0.22	0	0.42

6.4 Discussion and conclusion

The finding that hairF does not differ between COC-users and non-users suggests that the use of COCs containing EE and DRSP do not have an effect on long-term free cortisol levels. These results are in line with the findings of unaltered urinary cortisol after three weeks of EE/DRSP use by Ahmed *et al.* (2011), as well as unaltered plasma cortisol levels after three months of EE/DRSP use reported by De Leo *et al.* (2007), but are inconsistent with EE/DRSP-related increased serum cortisol seen by Macut *et al.* (2015). It is important to note that these studies depict the known EE-associated increase in CBG and total cortisol rather than free cortisol (Wiegratz *et al.*, 2003a) as is portrayed by hairF (Russell *et al.*, 2012). As explained in Section 2.8.2, DRSP and EE have been found to have differential effects on cortisol levels. DRSP might balance out the EE-related total cortisol increase to some degree through control of synthesis pathways (De Leo *et al.*, 2007; Louw-du Toit *et al.*, 2016). In support of this, the total glucocorticoids, reflecting the production of total free cortisol before a portion is converted to cortisone, was unaffected by EE/DRSP use in this study. In a study by Klipping *et al.* (2021), elevated CBG after six months of EE/DRSP use led to a slight decrease in calculated free cortisol (Klipping *et al.*, 2021). The authors also reported a comparable decrease with EE/LNG use (Klipping *et al.*, 2021). Similarly, Aleknaviciute *et al.* (2017) found lower hairF in EE/LNG users compared to controls (EE/DRSP was used for a minimum of four months). It is unclear why the results of the current study do not align with these findings. A possible explanation may be that different COC compositions might have varying effects on CBG binding capacity (Nenke *et al.*, 2017; Wiegratz *et al.*, 2003a) not only making calculations of free cortisol complex (Meyer *et al.*, 2016), but also creating disparity between measured free cortisol for different COCs . Nevertheless, similar changes in free cortisol between EE/DRSP and EE/LNG, yet contrasting changes in lipid profiles (HDL/LDL-cholesterol and triglycerides) (Klipping *et al.*, 2021; Lete *et al.*, 2015), suggest that the metabolic consequences of EE/DRSP are not mediated by cortisol levels, but rather by its activity, which was investigated in this study through the hairF/E ratio. The hairF/E ratio was not affected by long term EE/DRSP use. Although hairF/E is useful as

indication of overall 11β-HSD enzyme control on cortisol activity, it does not give an indication of cortisol activity within glucocorticoid-responsive tissues where stress related-illnesses originate. The limited research on the effects of estrogens and progestins on 11β-HSD1 in glucocorticoid tissues suggests that in addition to contrasting outcomes on different glucocorticoid tissues (liver and adipose tissue), estrogens and DRSP could counteract each other in the same tissues (Mattsson & Olsson, 2007; Stapelfeld & Maser, 2017). The lack of EE/DRSP-altered overall 11β-HSD activity in this study warrants more research to determine whether local 11β-HSD activity in various tissues is influenced by COCs. Altered 11β-HSD activity in the liver and adipose tissue can be investigated to understand the association between COC use and insulin resistance (Lete *et al.*, 2015), while investigating 11β-HSD2 activity in the kidneys may partly elucidate the mechanism by which EE/DRSP use leads to hypertension (Venter *et al.*, 2021) despite DRSP's lack of mineralocorticoid activity (Oelkers, 2004). Another possible mechanism through which COCs might contribute to stress-related illnesses, is reduced anti-glucocorticoid action of DHEA which could unfortunately not be assessed in this study.

The hairF/E ratio was significantly higher in Black Africans compared to Caucasians. No significant difference in any of the other stress markers investigated, indicates that while glucocorticoid secretion does not vary markedly between Black Africans and Caucasians, cortisol exposure is greater in Black Africans through variation in overall 11β-HSD activity. This could explain higher blood pressure and greater risk of metabolic syndrome in Black South Africans despite lower serum cortisol than Caucasian South African women (Schutte & Olckers, 2007). Although the means do not differ significantly, the larger standard deviation of all variables investigated within the Black African sample group, suggest that ethnicity should still be assessed as a covariate in future studies in the South African population. Further research is needed to determine if the variation in 11β-HSD activity between ethnicities is environmental or genetic. Although body fat percentage did not directly account for variation of hair stress markers amongst Black South Africans, investigation of environment or lifestyle factors that are likely to influence body fat percentage might explain this variation.

CHAPTER 7 CONCLUSION AND FUTURE DIRECTIONS

7.1 Summary

The aim of this study was to determine whether long term use of COCs containing EE and DRSP resulted in chronic biological stress through the evaluation of stress biomarkers in hair. A secondary aim was to assess ethnicity as a covariate of hair stress markers. To reach these aims an in-house LC-MS/MS method had to be developed to simultaneously quantify cortisol, cortisone and DHEA in hair and the method had to be validated before quantification of the analytes in hair samples. Two methods were successfully developed. However, each had limitations regarding different analytes. The selected method was validated, yielding acceptable validation results for cortisol and cortisone, but not DHEA. Poor accuracy and precision led to the unfortunate exclusion of DHEA from the study.

Application of the method in the sample groups revealed that none of the biomarkers were statistically different between Caucasian users and non-users, and that a higher hairF/E ratio was the only significant difference between Black Africans and Caucasians. Therefore, the long-term use of EE/DRSP-containing COCs does not lead to chronic biological stress. Furthermore, in future studies concerning the South African population, ethnicity should be considered and accounted for as a covariate of the hairF/E ratio and potentially also for hairF or hairE alone.

7.2 Advantages and shortcomings of study

The analysis of multiple analytes and their relationships allowed for a wholistic representation of total glucocorticoid activity in the body, which had not yet been investigated in COC users or different ethnic groups. Only COCs consisting of EE and DRSP were investigated and only in Caucasians. The findings of this study are thus not necessarily applicable to any other COC compositions or ethnicities.

Two methods were developed of which the most suitable one could be selected. Unfortunately, neither method was better for all analytes, but with improvements both methods can be further developed for routine use in the laboratory.

Stable isotopes were available for all analytes, allowing not only for accurate quantification but also sensitivity enhancement. Insufficient literature on correct use of isotopes for carrier effect was however problematic for DHEA quantification.

7.3 Future directions

7.3.1 Method improvements:

1. Optimisation of stable isotope concentrations may enhance the sensitivity of problematic analytes in both developed methods. Increasing cortisol-d_4 can enhance the carrier effect in the derivatised method, whereas decreasing the DHEA-d_2 concentration would minimise the cross-contribution by unlabelled analyte in the stable isotope. Alternatively, a DHEA stable isotope that is labelled with a larger deuterium number can be used.

2. Sample clean-up by SPE after derivatisation to remove excess derivatisation agent would allow for a larger injection volume and improved sensitivity.

3. Adding qualifier ions would improve the selectivity of both methods, especially for DHEA which has closely eluting peaks with the same MRM transition.

4. Post column infusion of ammonium fluoride will extend the column lifetime.

7.3.2 Future studies

1. Simultaneous assessment of hair cortisol, and DHEA as well as their ratios in chronic stress studies would be valuable in further elucidation of the aetiology of stress-related illnesses.

2. Investigation of the tissue-level effects of COCs on 11β-HSD can aid in understanding the mechanisms whereby COCs contribute to development of stress related-illnesses.

3. As previously mentioned, the results of this study are not directly translatable to other COC compositions. Evaluation of the stress markers assessed in this study, in combination with the above-mentioned considerations for different COCs can establish which COCs are associated with greater risk for stress related illnesses. This can also aid in safety profile assessment of newly developed COC compositions.

4. To establish whether a higher hairF/E ratio in Black South African women explains their elevated risk of stress related illnesses, the association between the cortisol/cortisone ratio and symptoms of these illnesses should be examined.

5. Determining why 11β-HSD activity differs between Black Africans and Caucasians may enable implementation of strategies to lower their risk of stress related illnesses.

BIBLIOGRAPHY

Abell, J.G., Stalder, T., Ferrie, J.E., Shipley, M.J., Kirschbaum, C., Kivimaki, M. & Kumari, M. 2016. Assessing cortisol from hair samples in a large observational cohort: The Whitehall II study. *Psychoneuroendocrinology*, 73:148-156. https://doi.org/10.1016/j.psyneuen.2016.07.214

Ågren, U.M., Anttila, M., Mäenpää-Liukko, K., Rantala, M.L., Rautiainen, H., Sommer, W.F. & Mommers, E. 2011. Effects of a monophasic combined oral contraceptive containing nomegestrol acetate and 17beta-oestradiol in comparison to one containing levonorgestrel and ethinylestradiol on markers of endocrine function. *The European Journal of Contraception & Reproductive Health Care*, 16(6):458-467. https://doi.org/10.3109/13625187.2011.614363

Ahmed, A.H., Gordon, R.D., Taylor, P.J., Ward, G., Pimenta, E. & Stowasser, M. 2011. Effect of contraceptives on aldosterone/renin ratio may vary according to the components of contraceptive, renin assay method, and possibly route of administration. *The Journal of Clinical Endocrinology & Metabolism*, 96(6):1797-1804. https://doi.org/10.1210/jc.2010-2918

Aleknaviciute, J., Tulen, J.H.M., De Rijke, Y.B., Bouwkamp, C.G., van der Kroeg, M., Timmermans, M., . . . Kushner, S.A. 2017. The levonorgestrel-releasing intrauterine device potentiates stress reactivity. *Psychoneuroendocrinology*, 80:39-45. https://doi.org/10.1016/j.psyneuen.2017.02.025

Bachmann, G. & Kopacz, S. 2009. Drospirenone/ethinyl estradiol 3 mg/20 µg (24/4 day regimen): hormonal contraceptive choices–use of a fourth-generation progestin. *Patient Preference and Adherence*, 3:259. https://doi.org/10.2147%2Fppa.s3901

Bae, Y.J. & Kratzsch, J. 2015. Corticosteroid-binding globulin: modulating mechanisms of bioavailability of cortisol and its clinical implications. *Best Practice & Reasearch Clinical Endocrinology & metabolism*, 29(5):761-772. https://doi.org/10.1016/j.beem.2015.09.001

Baier, F.S., Grandin, T., Engle, T.E., Archibeque, S.L., Wagner, J.J. & Edwards-Callaway, L.N. 2020. Effects of liver abscess presence on stress-related physiological parameters associated with well-being in beef feedlot cattle. *Applied Animal Science*, 36(3):437-446. https://doi.org/10.15232/aas.2019-01942

Balazs, Z., Schweizer, R.A.S., Frey, F.J., Rohner-Jeanrenaud, F. & Odermatt, A. 2008. DHEA induces 11β-HSD2 by acting on CCAAT/enhancer-binding proteins. *Journal of the American Society of Nephrology*, 19(1):92-101. https://doi.org/10.1681%2FASN.2007030263

Binz, T.M., Braun, U., Baumgartner, M.R. & Kraemer, T. 2016. Development of an LC–MS/MS method for the determination of endogenous cortisol in hair using 13C3-labeled cortisol as surrogate analyte. *Journal of Chromatography B*, 1033:65-72. https://doi.org/10.1016/j.jchromb.2016.07.041

Boadu, I. 2022. Coverage and determinants of modern contraceptive use in sub-Saharan Africa: further analysis of demographic and health surveys. *Reproductive Health*, 19(1):1-11. https://doi.org/10.1186%2Fs12978-022-01332-x

Breuner, C.W., Beyl, H.E. & Malisch, J.L. 2020. Corticosteroid-binding globulins: Lessons from biomedical research. *Molecular and Cellular Endocrinology*, 514:110857. https://doi.org/10.1016/j.mce.2020.110857

Burton, P.J., Krozowski, Z.S. & Waddell, B.J. 1998. Immunolocalization of 11beta-hydroxysteroid dehydrogenase types 1 and 2 in rat uterus: variation across the estrous cycle and regulation by estrogen and progesterone. *Endocrinology*, 139(1):376-382. https://doi.org/10.1210/endo.139.1.5692

Cain, D.W. & Cidlowski, J.A. 2017. Immune regulation by glucocorticoids. *Nature Reviews Endocrinology*, 17(4):233-247. https://doi.org/10.1038/nri.2017.1

Carroll, D., Phillips, A.C., Lord, J.M., Arlt, W. & Batty, G.D. 2011. Cortisol, dehydroepiandrosterone sulphate, their ratio and hypertension: evidence of associations in male veterans from the Vietnam Experience Study. *Journal of Human Hypertension*, 25(7):418-424. https://doi.org/10.1038/jhh.2011.6

Chen, Z., Li, J., Zhang, J., Xing, X., Gao, W., Lu, Z. & Deng, H. 2013. Simultaneous determination of hair cortisol, cortisone and DHEAS with liquid chromatography-electrospray ionization-tandem mass spectrometry in negative mode. *Journal of Chromatography B*, 929:187-194. https://doi.org/10.1016/j.jchromb.2013.04.026

Chen, Z., Li, J., Xu, G., Yang, J., Zhang, J. & Deng, H. 2014. Simultaneous measurements of cortisol and cortisone in urine and hair for the assessment of 11β-hydroxysteroid dehydrogenase activity among methadone maintenance treatment patients with LC-ESI–MS/MS. *Journal of Chromatography B*, 969:77-84. https://doi.org/10.1016/j.jchromb.2014.08.001

Chen, Z., Zhang, Q., Chen, S., Wang, W., Liu, G. & Deng, H. 2019. Determination, intercorrelation and intraindividual stability of five steroids in hair, saliva and urine among Chinese college students. *Steroids*, 149:108418. https://doi.org/10.1016/j.steroids.2019.05.010

Cole, T., Short, K.L. & Hooper, S.B. 2019. The science of steroids. *Seminars in Fetal and Neonatal Medicine*, 24(3):170-175. https://doi.org/10.1016/j.siny.2019.05.005

Condon, E.M., Holland, M.L., Slade, A., Redeker, N.S., Mayes, L.C. & Sadler, L. 2019. Associations between maternal experiences of discrimination and biomarkers of toxic stress in school-aged children. *Maternal and Child Health Journal*, 23(9):1147-1151. https://doi.org/10.1007/s10995-019-02779-4

Cooper, M.S. & Stewart, P.M. 2009. 11Beta-hydroxysteroid dehydrogenase type 1 and its role in the hypothalamus-pituitary-adrenal axis, metabolic syndrome, and inflammation. *The Journal of Clinical Endocrinology & Metabolism*, 94(12):4645-4654. https://doi.org/10.1210/jc.2009-1412

De Groote, D., Perrier d'Hauterive, S., Pintiaux, A., Balteau, B., Gerday, C., Claesen, J. & Foidart, J.M. 2009. Effects of oral contraception with ethinylestradiol and drospirenone on oxidative stress in women 18-35 years old. *Contraception*, 80(2):187-193. https://doi.org/10.1016/j.contraception.2009.02.015

de la Torre, X., Iannone, M. & Botre, F. 2021. Improving the detection of anabolic steroid esters in human serum by LC-MS. *Journal of Pharmaceutical and Biomedical Analysis*, 194:113807. https://doi.org/10.1016/j.jpba.2020.113807

De Leo, V., Morgante, G., Piomboni, P., Musacchio, M.C., Petraglia, F. & Cianci, A. 2007. Evaluation of effects of an oral contraceptive containing ethinylestradiol combined with drospirenone on adrenal steroidogenesis in hyperandrogenic women with polycystic ovary syndrome. *Fertility and Sterility*, 88(1):113-117. https://doi.org/10.1016/j.fertnstert.2006.11.137

de Wit, A.E., Booij, S.H., Giltay, E.J., Joffe, H., Schoevers, R.A. & Oldehinkel, A.J. 2020. Association of use of oral contraceptives with depressive symptoms among adolescents and young women. *JAMA Psychiatry*, 77(1):52-59. https://doi.org/doi:10.1001/jamapsychiatry.2019.2838

Dong, T., Cheng, Y.W., Yang, F., Sun, P.W., Zhu, C.J., Zhu, L. & Zhang, G.X. 2015. Chronic Stress Facilitates the Development of Deep Venous Thrombosis. *Oxidative Medicine and Cellular Longevity*, 2015:384535. https://doi.org/10.1155/2015/384535

Dong, Z., Wang, C., Zhang, J. & Wang, Z. 2017. A UHPLC-MS/MS method for profiling multifunctional steroids in human hair. *Analytical and Bioanalytical Chemistry*, 409(20):4751-4769. https://doi.org/10.1007/s00216-017-0419-2

Dragoman, M.V. 2014. The combined oral contraceptive pill-recent developments, risks and benefits. *Best Practice & Research Clinical Obstetrics & Gynaecology*, 28(6):825-834. https://doi.org/10.1016/j.bpobgyn.2014.06.003

Eick, C., Klinger-König, J., Zylla, S., Hannemann, A., Budde, K., Henning, A.K., . . . Hertel, J. 2021. Broad Metabolome Alterations Associated with the Intake of Oral Contraceptives Are Mediated by Cortisol in Premenopausal Women. *Metabolites*, 11(4):193. https://doi.org/10.3390/metabo11040193

Fernández, R.D.V., Diaz, A., Bongiovanni, B., Gallucci, G., Bértola, D., Gardeñez, W., . . . D'Attilio, L. 2020. Evidence for a More Disrupted Immune-Endocrine Relation and Cortisol Immunologic Influences in the Context of Tuberculosis and Type 2 Diabetes Comorbidity. *Frontiers in Endocrinology*, 11:126. https://doi.org/10.3389/fendo.2020.00126

Fischer, S., Duncko, R., Hatch, S.L., Papadopoulos, A., Goodwin, L., Frissa, S., . . . Cleare, A.J. 2017. Sociodemographic, lifestyle, and psychosocial determinants of hair cortisol in a South London community sample. *Psychoneuroendocrinology*, 76:144-153. https://doi.org/10.1016/j.psyneuen.2016.11.011

Forman, H.J. & Zhang, H. 2021. Targeting oxidative stress in disease: Promise and limitations of antioxidant therapy. *Nature Reviews Drug Discovery*, 20(9):689-709. https://doi.org/10.1038%2Fs41573-021-00233-1

Gallo-Payet, N., Martinez, A. & Lacroix, A. 2017. Editorial: ACTH Action in the Adrenal Cortex: From Molecular Biology to Pathophysiology. *Frontiers in endocrinology*, 8:101. https://doi.org/10.3389/fendo.2017.00101

Gao, W., Stalder, T., Foley, P., Rauh, M., Deng, H. & Kirschbaum, C. 2013. Quantitative analysis of steroid hormones in human hair using a column-switching LC-APCI-MS/MS assay. *Journal of Chromatography B*, 928:1-8. https://doi.org/10.1016/j.jchromb.2013.03.008

Gao, W., Kirschbaum, C., Grass, J. & Stalder, T. 2016. LC-MS based analysis of endogenous steroid hormones in human hair. *The Journal of Steroid Biochemistry and Molecular Biology*, 162:92-99. https://doi.org/10.1016/j.jsbmb.2015.12.022

Garland, W.A. & Barbalas, M.P. 1986. Applications to analytic chemistry: an evaluation of stable isotopes in mass spectral drug assays. *The Journal of Clinical Pharmacology*, 26(6):412-418. https://doi.org/10.1002/j.1552-4604.1986.tb03550.x

Gaudl, A., Kratzsch, J., Bae, Y.J., Kiess, W., Thiery, J. & Ceglarek, U. 2016. Liquid chromatography quadrupole linear ion trap mass spectrometry for quantitative steroid hormone analysis in plasma, urine, saliva and hair. *Journal of Chromatography A*, 1464:64-71. https://doi.org/10.1016/j.chroma.2016.07.087

George, R., Haywood, A., Khan, S., Radovanovic, M., Simmonds, J. & Norris, R. 2018. Enhancement and Suppression of Ionization in Drug Analysis Using HPLC-MS/MS in Support of Therapeutic Drug Monitoring: A Review of Current Knowledge of Its Minimization and Assessment. *Therapeutic Drug Monitoring*, 40(1):1-8. https://doi.org/10.1097/FTD.0000000000000471

González, O., Blanco, M.E., Iriarte, G., Bartolomé, L., Maguregui, M.I. & Alonso, R.M. 2014. Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect. *Journal of Chromatography A*, 1353:10-27. https://doi.org/10.1016/j.chroma.2014.03.077

Greff, M.J.E., Levine, J.M., Abuzgaia, A.M., Elzagallaai, A.A., Rieder, M.J. & van Uum, S.H.M. 2019. Hair cortisol analysis: An update on methodological considerations and clinical applications. *Clinical Biochemistry*, 63:1-9. https://doi.org/10.1016/j.clinbiochem.2018.09.010

Handa, R.J. & Weiser, M.J. 2014. Gonadal steroid hormones and the hypothalamo-pituitary-adrenal axis. *Frontiers in Neuroendocrinology*, 35(2):197-220. https://doi.org/10.1016/j.yfrne.2013.11.001

Hazell, G., Horn, G., Lightman, S.L. & Spiga, F. 2019. Dynamics of ACTH-Mediated Regulation of Gene Transcription in ATC1 and ATC7 Adrenal Zona Fasciculata Cell Lines. *Endocrinology*, 160(3):587-604. https://doi.org/10.1210/en.2018-00840

Henley, D., Lightman, S. & Carrell, R. 2016. Cortisol and CBG—getting cortisol to the right place at the right time. *Pharmacology & Therapeutics*, 166:128-135. https://doi.org/10.1016/j.pharmthera.2016.06.020

Herrera, A.Y., Faude, S., Nielsen, S.E., Locke, M. & Mather, M. 2019. Effects of hormonal contraceptive phase and progestin generation on stress-induced cortisol and progesterone release. *Neurobiology of Stress*, 10:100151. https://doi.org/10.1016/j.ynstr.2019.100151

Hertel, J., König, J., Homuth, G., Van der Auwera, S., Wittfeld, K., Pietzner, M., . . . Grabe, H.J. 2017. Evidence for Stress-like Alterations in the HPA-Axis in Women Taking Oral Contraceptives. *Scientific Reports*, 7(1):14111. https://doi.org/10.1038/s41598-017-13927-7

Higashi, T., Nishio, T., Hayashi, N. & Shimada, K. 2007. Alternative procedure for charged derivatization to enhance detection responses of steroids in electrospray ionization-MS. *Chemical and Pharmaceutical Bulletin*, 55(4):662-665. https://doi.org/10.1248/cpb.55.662

Higashi, T. & Ogawa, S. 2016. Chemical derivatization for enhancing sensitivity during LC/ESI-MS/MS quantification of steroids in biological samples: a review. *The Journal of Steroid Biochemistry and Molecular Biology*, 162:57-69. https://doi.org/10.1016/j.jsbmb.2015.10.003

Hobo, Y., Nishikawa, J., Miyashiro, Y. & Fujikata, A. 2020. Measurement of steroid hormones by liquid chromatography-tandem mass spectrometry with small amounts of hair. *Steroids*, 164:108732. https://doi.org/10.1016/j.steroids.2020.108732

Hobo, Y., Nishikawa, J., Miyashiro, Y. & Fujikata, A. 2021. Analysis of Hair Steroid Hormone Concentrations at Different Parts of the Head by Liquid Chromatography-Tandem Mass Spectrometry. *Clinica Chimica Acta* https://doi.org/10.1016/j.cca.2021.10.004

Honour, J.W. 2011. Development and validation of a quantitative assay based on tandem mass spectrometry. *Annals of Clinical Biochemistry*, 48(Pt 2):97-111. https://doi.org/10.1258/acb.2010.010176

Hothorn, L.A. 2016. The two-step approach—a significant ANOVA F-test before Dunnett's comparisons against a control—is not recommended. *Communications in Statistics-Theory and Methods*, 45(11):3332-3343. https://doi.org/10.1080/03610926.2014.902225

Hucklebridge, F., Hussain, T., Evans, P. & Clow, A. 2005. The diurnal patterns of the adrenal steroids cortisol and dehydroepiandrosterone (DHEA) in relation to awakening. *Psychoneuroendocrinology*, 30(1):51-57. https://doi.org/10.1016/j.psyneuen.2004.04.007

Ilg, L., Kirschbaum, C., Li, S.C., Wimberger, P., Nitzsche, K., Rosenlocher, F. & Alexander, N. 2020. No Association of Antenatal Synthetic Glucocorticoid Exposure and Hair Steroid Levels in Children and Adolescents. *The Journal of Clinical Endocrinology & Metabolism*, 105(1):e575-e582. https://doi.org/10.1210/clinem/dgz064

National Center for Biotechnology Information. 2023. PubChem Coumpound Summary for CID 25546, Ammonium fluoride. https://pubchem.ncbi.nlm.nih.gov/compound/Ammonium-fluoride Date of access: 18 September 2023.

Iob, E. & Steptoe, A. 2019. Cardiovascular Disease and Hair Cortisol: a Novel Biomarker of Chronic Stress. *Current Cardiology Reports*, 21(10):116. https://doi.org/10.1007/s11886-019-1208-7

Jagannathan, R., Patel, S.A., Ali, M.K. & Narayan, K.M.V. 2019. Global Updates on Cardiovascular Disease Mortality Trends and Attribution of Traditional Risk Factors. *Current Diabetes Reports*, 19(7):44. https://doi.org/10.1007/s11892-019-1161-2

Jeckel, C.M., Lopes, R.P., Berleze, M.C., Luz, C., Feix, L., Argimon, II, . . . Bauer, M.E. 2010. Neuroendocrine and immunological correlates of chronic stress in 'strictly healthy' populations. *Neuroimmunomodulation*, 17(1):9-18. https://doi.org/10.1159/000243080

Joca, S.R., Ferreira, F.R. & Guimaraes, F.S. 2007. Modulation of stress consequences by hippocampal monoaminergic, glutamatergic and nitrergic neurotransmitter systems. *Stress*, 10(3):227-249. https://doi.org/10.1080/10253890701223130

Juck, M. & Long, W. 2017. Determination of Hormones in Serum by LC/MS/MS Using Agilent Bond Elut Plexa SPE; Application note, Agilent Technnologies, Inc. Publication number 5991-8042EN.

Kadian, N., Raju, K.S., Rashid, M., Malik, M.Y., Taneja, I. & Wahajuddin, M. 2016. Comparative assessment of bioanalytical method validation guidelines for pharmaceutical industry. *Journal of Pharmaceutical and Biomedical Analysis*, 126:83-97. https://doi.org/10.1016/j.jpba.2016.03.052

Kamin, H.S. & Kertes, D.A. 2017. Cortisol and DHEA in development and psychopathology. *Hormones and Behavior*, 89:69-85. https://doi.org/10.1016/j.yhbeh.2016.11.018

Kataoka, H., Ehara, K., Yasuhara, R. & Saito, K. 2013. Simultaneous determination of testosterone, cortisol, and dehydroepiandrosterone in saliva by stable isotope dilution on-line in-tube solid-phase microextraction coupled with liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, 405(1):331-340. https://doi.org/10.1007/s00216-012-6479-4

Keevil, B.G. 2016. LC-MS/MS analysis of steroids in the clinical laboratory. *Clinical Biochemistry*, 49(13-14):989-997. https://doi.org/10.1016/j.clinbiochem.2016.04.009

Kirschbaum, C., Tietze, A., Skoluda, N. & Dettenborn, L. 2009. Hair as a retrospective calendar of cortisol production—increased cortisol incorporation into hair in the third trimester of pregnancy. *Psychoneuroendocrinology*, 34(1):32-37. https://doi.org/10.1016/j.psyneuen.2008.08.024

Klipping, C., Duijkers, I., Mawet, M., Maillard, C., Bastidas, A., Jost, M. & Foidart, J.M. 2021. Endocrine and metabolic effects of an oral contraceptive containing estetrol and drospirenone. *Contraception*, 103(4):213-221. https://doi.org/10.1016/j.contraception.2021.01.001

Kudielka, B.M., Gierens, A., Hellhammer, D.H., Wüst, S. & Schlotz, W. 2012. Salivary cortisol in ambulatory assessment—some dos, some don'ts, and some open questions. *Psychosomatic Medicine*, 74(4):418-431. https://doi.org/10.1097/psy.0b013e31825434c7

Kuo, T., McQueen, A., Chen, T.C. & Wang, J.C. 2015. Regulation of Glucose Homeostasis by Glucocorticoids. *Advances in Experimental Medicine and Biology*, 872:99-126. https://doi.org/10.1007/978-1-4939-2895-8_5

Kushnir, M.M., Rockwood, A.L., Roberts, W.L., Yue, B., Bergquist, J. & Meikle, A.W. 2011. Liquid chromatography tandem mass spectrometry for analysis of steroids in clinical laboratories. *Clinical Biochemistry*, 44(1):77-88. https://doi.org/10.1016/j.clinbiochem.2010.07.008

Lee, D.B., Peckins, M.K., Miller, A.L., Hope, M.O., Neblett, E.W., Assari, S., . . . Zimmerman, M.A. 2021. Pathways from racial discrimination to cortisol/DHEA imbalance: protective role of religious involvement. *Ethnicity and Health*, 26(3):413-430. https://doi.org/10.1080/13557858.2018.1520815

Lee, S. & Lee, D.K. 2018. What is the proper way to apply the multiple comparison test? *Korean Journal of Anesthesiology*, 71(5):353-360. https://doi.org/10.4097%2Fkja.d.18.00242

Lehrer, H.M., Dubois, S.K., Maslowsky, J., Laudenslager, M.L. & Steinhardt, M.A. 2016. Hair cortisol concentration and glycated hemoglobin in African American adults. *Psychoneuroendocrinology*, 72:212-218. https://doi.org/10.1016/j.psyneuen.2016.06.018

Lehrer, H.M., Goosby, B.J., Dubois, S.K., Laudenslager, M.L. & Steinhardt, M.A. 2020. Race moderates the association of perceived everyday discrimination and hair cortisol concentration. *Stress*, 23(5):529-537. https://doi.org/10.1080/10253890.2019.1710487

Lete, I., Chabbert-Buffet, N., Jamin, C., Lello, S., Lobo, P., Nappi, R.E. & Pintiaux, A. 2015. Haemostatic and metabolic impact of estradiol pills and drospirenone-containing ethinylestradiol pills vs. levonorgestrel-containing ethinylestradiol pills: A literature review. *The European Journal of Contraception & Reproductive Health Care*, 20(5):329-343. https://doi.org/10.3109/13625187.2015.1050091

Lightman, S.L., Birnie, M.T. & Conway-Campbell, B.L. 2020. Dynamics of ACTH and Cortisol Secretion and Implications for Disease. *Endocrine Reviews*, 41(3):470-490. https://doi.org/10.1210/endrev/bnaa002

Lindner, J.M., Vogeser, M. & Grimm, S.H. 2017. Biphenyl based stationary phases for improved selectivity in complex steroid assays. *Journal of Pharmaceutical and Biomedical Analysis*, 142:66-73. https://doi.org/10.1016/j.jpba.2017.04.020

Loussouarn, G., Lozano, I., Panhard, S., Collaudin, C., El Rawadi, C. & Genain, G. 2016. Diversity in human hair growth, diameter, colour and shape. An in vivo study on young adults from 24 different ethnic groups observed in the five continents. *European Journal of Dermatology*, 26(2):144-154. https://doi.org/10.1684/ejd.2015.2726

Louw-du Toit, R., Perkins, M.S., Snoep, J.L., Storbeck, K.H. & Africander, D. 2016. Fourth-Generation Progestins Inhibit 3beta-Hydroxysteroid Dehydrogenase Type 2 and Modulate the Biosynthesis of Endogenous Steroids. *PLoS One*, 11(10):e0164170. https://doi.org/10.1371/journal.pone.0164170

Louw-du Toit, R., Storbeck, K.H., Cartwright, M., Cabral, A. & Africander, D. 2017. Progestins used in endocrine therapy and the implications for the biosynthesis and metabolism of endogenous steroid hormones. *Molecular and Cellular Endocrinology*, 441:31-45. https://doi.org/10.1016/j.mce.2016.09.004

Macut, D., Bozic Antić, I., Nestorov, J., Topalović, V., Bjekic Macut, J., Panidis, D., . . . Vojnovic Milutinovic, D. 2015. The influence of combined oral contraceptives containing drospirenone on hypothalamic-pituitary-adrenocortical axis activity and glucocorticoid receptor expression and function in women with polycystic ovary syndrome. *Hormones*, 14(1):109-117. https://doi.org/10.14310/horm.2002.1526

Maninger, N., Wolkowitz, O.M., Reus, V.I., Epel, E.S. & Mellon, S.H. 2009. Neurobiological and neuropsychiatric effects of dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS). *Frontiers in Endocrinology*, 30(1):65-91. https://doi.org/10.1016/j.yfrne.2008.11.002

Marcos, J. & Pozo, O.J. 2016. Current LC-MS methods and procedures applied to the identification of new steroid metabolites. *The Journal of Steroid Biochemistry and Molecular Biology*, 162:41-56. https://doi.org/10.1016/j.jsbmb.2015.12.012

Marvar, P.J., Vinh, A., Thabet, S., Lob, H.E., Geem, D., Ressler, K.J. & Harrison, D.G. 2012. T lymphocytes and vascular inflammation contribute to stress-dependent hypertension. *Biological Psychiatry*, 71(9):774-782. https://doi.org/10.1016/j.biopsych.2012.01.017

Mattsson, C. & Olsson, T. 2007. Estrogens and glucocorticoid hormones in adipose tissue metabolism. *Current Medicinal Chemistry*, 14(27):2918-2924. https://doi.org/10.2174/092986707782359972

McEwen, B.S. 1998. Stress, adaptation, and disease. Allostasis and allostatic load. *Annals of the New York Academy of Sciences*, 840(1):33-44. https://doi.org/10.1111/j.1749-6632.1998.tb09546.x

McHugh, M.L. 2011. Multiple comparison analysis testing in ANOVA. *Biochemica Medica*, 21(3):203-209. https://doi.org/10.11613/bm.2011.029

McNair, H. & Polite, L.N. 2007. 17 Troubleshooting in high performance liquid chromatography. *In:* Ahuja, S. & Rasmussen, H., *eds*. *HPLC Method Development for Pharmaceuticals*. 1st ed: Elsevier. pp. 459-477.

Meyer, E.J., Nenke, M.A., Rankin, W., Lewis, J.G. & Torpy, D.J. 2016. Corticosteroid-Binding Globulin: A Review of Basic and Clinical Advances. *Hormone and Metabolic Research*, 48(6):359-371. https://doi.org/10.1055/s-0042-108071

Miller, W.L. & Auchus, R.J. 2011. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine Reviews*, 32(1):81-151. https://doi.org/10.1210/er.2010-0013

Muller, C., Hennebert, O. & Morfin, R. 2006. The native anti-glucocorticoid paradigm. *The Journal of Steroid Biochemistry and Molecular Biology*, 100(1-3):95-105. https://doi.org/10.1016/j.jsbmb.2006.03.001

Musana, J.W., Cohen, C.R., Kuppermann, M., Gerona, R., Wanyoro, A., Aguilar, D., . . . Weiss, S.J. 2020. Association of differential symptoms of stress to hair cortisol and cortisone concentrations among pregnant women in Kenya. *Stress*, 23(5):556-566. https://doi.org/10.1080/10253890.2019.1696305

Nadarajah, N., Skadberg, Ø., Adaway, J. & Brede, C. 2017. Multiplexed analysis of steroid hormones in saliva by LC-MS/MS with 2-hydrazinopyridine derivatization. *Clinical Mass Spectrometry*, 4:1-10. https://doi.org/10.1016/j.clinms.2017.08.001

Nenke, M.A., Zeng, A., Meyer, E.J., Lewis, J.G., Rankin, W., Johnston, J., . . . Torpy, D.J. 2017. Differential Effects of Estrogen on Corticosteroid-Binding Globulin Forms Suggests Reduced Cleavage in Pregnancy. *Journal of the Endocrine Society*, 1(3):202-210. https://doi.org/10.1210/js.2016-1094

Ney, L.J., Felmingham, K.L., Bruno, R., Matthews, A. & Nichols, D.S. 2021. Chloroform-based liquid-liquid extraction and LC-MS/MS quantification of endocannabinoids, cortisol and progesterone in human hair. *Journal of Pharmaceutical and Biomedical Analysis*, 201:114103. https://doi.org/10.1016/j.jpba.2021.114103

Nicolaides, N.C., Kyratzi, E., Lamprokostopoulou, A., Chrousos, G.P. & Charmandari, E. 2015. Stress, the stress system and the role of glucocorticoids. *Neuroimmunomodulation*, 22(1-2):6-19. https://doi.org/10.1159/000362736

Nicolaides, N.C., Charmandari, E., Kino, T. & Chrousos, G.P. 2017. Stress-Related and Circadian Secretion and Target Tissue Actions of Glucocorticoids: Impact on Health. *Frontiers in Endocrinology*, 8:70. https://doi.org/10.3389/fendo.2017.00070

Noppe, G., de Rijke, Y.B., Dorst, K., van den Akker, E.L. & van Rossum, E.F. 2015. LC-MS/MS-based method for long-term steroid profiling in human scalp hair. *Clinical Endocrinology*, 83(2):162-166. https://doi.org/10.1111/cen.12781

Oelkers, W. 2004. Drospirenone, a progestogen with antimineralocorticoid properties: a short review. *Meolecular and Cellular Endocrinology*, 217(1-2):255-261. https://doi.org/10.1016/j.mce.2003.10.030

Papadopoulos, V. & Miller, W.L. 2012. Role of mitochondria in steroidogenesis. *Best Practice & Research Clinical Endocrinology & metabolism*, 26(6):771-790. https://doi.org/10.1016/j.beem.2012.05.002

Perogamvros, I., Keevil, B., Ray, D.W. & Trainer, P.J. 2010. Salivary cortisone is a potential biomarker for serum free cortisol. *The Journal of Clinical Endocrinology & Metabolism*, 95(11):4951-4958. https://doi.org/10.1210/jc.2010-1215

Petersen, B. & Vouros, P. 1977. Analysis of thyroid hormones as their heptafluorobutyryl methyl ester derivatives by gas chromatography-mass spectrometry. *Analytical Chemistry*, 49(9):1304-1311. https://doi.org/10.1021/ac50017a006

Phillips, A.C., Carroll, D., Gale, C.R., Lord, J.M., Arlt, W. & Batty, G.D. 2010. Cortisol, DHEAS, their ratio and the metabolic syndrome: evidence from the Vietnam Experience Study. *European Journal of Endocrinology*, 162(5):919-923. https://doi.org/10.1530/eje-09-1078

Polson, C., Sarkar, P., Incledon, B., Raguvaran, V. & Grant, R. 2003. Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B*, 785(2):263-275. https://doi.org/10.1016/S1570-0232(02)00914-5

Prall, S.P. & Muehlenbein, M.P. 2018. DHEA Modulates Immune Function: A Review of Evidence. *Vitamins and Hormones*, 108:125-144. https://doi.org/10.1016/bs.vh.2018.01.023

Pretorius, C.J., Galligan, J.P., McWhinney, B.C., Briscoe, S.E. & Ungerer, J.P. 2011. Free cortisol method comparison: ultrafiltation, equilibrium dialysis, tracer dilution, tandem mass spectrometry and calculated free cortisol. *Clinica Chimica Acta*, 412(11-12):1043-1047. https://doi.org/10.1016/j.cca.2011.02.019

Prough, R.A., Clark, B.J. & Klinge, C.M. 2016. Novel mechanisms for DHEA action. *Journal of Molecular endocrinology*, 56(3):R139-155. https://doi.org/10.1530/JME-16-0013

Qiao, S., Li, X., Zilioli, S., Chen, Z., Deng, H., Pan, J. & Guo, W. 2017. Hair measurements of cortisol, DHEA, and DHEA to cortisol ratio as biomarkers of chronic stress among people living with HIV in China: known-group validation. *PLoS One*, 12(1):e0169827. https://doi.org/10.1371%2Fjournal.pone.0169827

Quinete, N., Bertram, J., Reska, M., Lang, J. & Kraus, T. 2015. Highly selective and automated online SPE LC–MS3 method for determination of cortisol and cortisone in human hair as

biomarker for stress related diseases. *Talanta*, 134:310-316. https://doi.org/10.1016/j.talanta.2014.11.034

Raff, H. & Carroll, T. 2015. Cushing's syndrome: from physiological principles to diagnosis and clinical care. *The Journal of Physiology*, 593(3):493-506. https://doi.org/10.1113/jphysiol.2014.282871

Raul, J.S., Cirimele, V., Ludes, B. & Kintz, P. 2004. Detection of physiological concentrations of cortisol and cortisone in human hair. *Clinical Biochemistry*, 37(12):1105-1111. https://doi.org/10.1016/j.clinbiochem.2004.02.010

Regidor, P.A. & Schindler, A.E. 2017. Antiandrogenic and antimineralocorticoid health benefits of COC containing newer progestogens: dienogest and drospirenone. *Oncotarget*, 8(47):83334-83342. https://doi.org/10.18632/oncotarget.19833

Ringnér, M. 2008. What is principal component analysis? *Nature Biotechnology*, 26(3):303-304. https://doi.org/10.1038/nbt0308-303

Rippe, R.C., Noppe, G., Windhorst, D.A., Tiemeier, H., van Rossum, E.F., Jaddoe, V.W., . . . van den Akker, E.L. 2016. Splitting hair for cortisol? Associations of socio-economic status, ethnicity, hair color, gender and other child characteristics with hair cortisol and cortisone. *Psychoneuroendocrinology*, 66:56-64. https://doi.org/10.1016/j.psyneuen.2015.12.016

Rodrigo, R., Gonzalez, J. & Paoletto, F. 2011. The role of oxidative stress in the pathophysiology of hypertension. *Hypertension Research*, 34(4):431-440. https://doi.org/10.1038/hr.2010.264

Russell, E., Koren, G., Rieder, M. & Van Uum, S. 2012. Hair cortisol as a biological marker of chronic stress: current status, future directions and unanswered questions. *Psychoneuroendocrinology*, 37(5):589-601.

Russell, E., Kirschbaum, C., Laudenslager, M.L., Stalder, T., de Rijke, Y., van Rossum, E.F., . . . Koren, G. 2015. Toward standardization of hair cortisol measurement: results of the first international interlaboratory round robin. *Therapeutic Drug Monitoring*, 37(1):71-75. https://doi.org/10.1097/FTD.0000000000000148

Russell, G. & Lightman, S. 2019. The human stress response. *Nature Reviews Endocrinology*, 15(9):525-534. https://doi.org/10.1038/s41574-019-0228-0

Rychlik, M. 2011. Stable isotope dilution assays in vitamin analysis–A review of principles and applications. *In:* Rychlik, M., *ed. Fortified foods with vitamins: analytical concepts to assure better and safer products*. Wiley. pp. 1-19.

Sandrini, L., Ieraci, A., Amadio, P., Zarà, M. & Barbieri, S.S. 2020. Impact of Acute and Chronic Stress on Thrombosis in Healthy Individuals and Cardiovascular Disease Patients. *International Journal of Molecular Sciences*, 21(21):7818. https://doi.org/10.3390/ijms21217818

Santa, T. 2013. Derivatization in liquid chromatography for mass spectrometric detection. *Drug Discoveries & Therapeutics*, 7(1):9-17. https://doi.org/10.5582/ddt.2013.v7.1.9

Sato, K., Iemitsu, M., Aizawa, K. & Ajisaka, R. 2008. Testosterone and DHEA activate the glucose metabolism-related signaling pathway in skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism*, 294(5):E961-968. https://doi.org/10.1152/ajpendo.00678.2007

Sauder, D.C. & DeMars, C.E. 2019. An updated recommendation for multiple comparisons. *Advances in Methods and Practices in Psychological Science*, 2(1):26-44. https://doi.org/10.1177/2515245918808784

Savineau, J.P., Marthan, R. & Dumas de la Roque, E. 2013. Role of DHEA in cardiovascular diseases. *Biochemical Pharmacology*, 85(6):718-726. https://doi.org/10.1016/j.bcp.2012.12.004

Schiffer, L., Shaheen, F., Gilligan, L.C., Storbeck, K.H., Hawley, J.M., Keevil, B.G., . . . Taylor, A.E. 2022. Multi-steroid profiling by UHPLC-MS/MS with post-column infusion of ammonium fluoride. *Journal of Chromatography B*, 1209:123413. https://doi.org/10.1016/j.jchromb.2022.123413

Schutte, A.E. & Olckers, A. 2007. Metabolic syndrome risk in black South African women compared to Caucasian women. *Hormone and Metabolic Research*, 39(9):651-657. https://doi.org/10.1055/s-2007-985394

Short, S.J., Stalder, T., Marceau, K., Entringer, S., Moog, N.K., Shirtcliff, E.A., . . . Buss, C. 2016. Correspondence between hair cortisol concentrations and 30-day integrated daily salivary and weekly urinary cortisol measures. *Psychoneuroendocrinology*, 71:12-18. https://doi.org/10.1016/j.psyneuen.2016.05.007

Shrivastava, A. & Gupta, V.B. 2011. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of Young Scientists*

2(1):21-25.

Sitruk-Ware, R. & Nath, A. 2011. Metabolic effects of contraceptive steroids. *Reviews in Endocrine and Metabolic Disorders*, 12(2):63-75. https://doi.org/10.1007/s11154-011-9182-4

Smith, R.E., Salamonsen, L.A., Komesaroff, P.A., Li, K.X., Myles, K.M., Lawrence, M. & Krozowski, Z. 1997. 11β-Hydroxysteroid dehydrogenase type II in the human endometrium: localization and activity during the menstrual cycle. *The Journal of Clinical Endocrinology & Metabolism*, 82(12):4252-4257. https://doi.org/10.1210/jcem.82.12.4451

Smrekar, K. & Lodise, N.M. 2022. Combined Oral Contraceptive Use and Breast Cancer Risk: Select Considerations for Clinicians. *Nursing for Women's Health*, 26(3):242-249. https://doi.org/10.1016/j.nwh.2022.01.003

Son, H.H., Yun, W.S. & Cho, S.H. 2020. Development and validation of an LC-MS/MS method for profiling 39 urinary steroids (estrogens, androgens, corticoids, and progestins). *Biomedical Chromatography*, 34(2):e4723. https://doi.org/10.1002/bmc.4723

Sosvorova, L., Vitku, J., Chlupacova, T., Mohapl, M. & Hampl, R. 2015. Determination of seven selected neuro- and immunomodulatory steroids in human cerebrospinal fluid and plasma using LC-MS/MS. *Steroids*, 98:1-8. https://doi.org/10.1016/j.steroids.2015.01.019

Spencer, R.L. & Deak, T. 2017. A users guide to HPA axis research. *Physiology & Behavior*, 178:43-65. https://doi.org/10.1016/j.physbeh.2016.11.014

Stalder, T. & Kirschbaum, C. 2012. Analysis of cortisol in hair--state of the art and future directions. *Brain, Behavior, and Immunity*, 26(7):1019-1029. https://doi.org/10.1016/j.bbi.2012.02.002

Stalder, T., Steudte, S., Miller, R., Skoluda, N., Dettenborn, L. & Kirschbaum, C. 2012. Intraindividual stability of hair cortisol concentrations. *Psychoneuroendocrinology*, 37(5):602-610. https://doi.org/10.1016/j.psyneuen.2011.08.007

Stalder, T., Kirschbaum, C., Kudielka, B.M., Adam, E.K., Pruessner, J.C., Wust, S., . . . Clow, A. 2016. Assessment of the cortisol awakening response: Expert consensus guidelines. *Psychoneuroendocrinology*, 63:414-432. https://doi.org/10.1016/j.psyneuen.2015.10.010

Stapelfeld, C. & Maser, E. 2017. Sex hormones reduce NNK detoxification through inhibition of short-chain dehydrogenases/reductases and aldo-keto reductases in vitro. *Chemico-Biological Interactions*, 276:167-173. https://doi.org/10.1016/j.cbi.2017.02.016

Staufenbiel, S.M., Penninx, B.W., de Rijke, Y.B., van den Akker, E.L. & van Rossum, E.F. 2015. Determinants of hair cortisol and hair cortisone concentrations in adults. *Psychoneuroendocrinology*, 60:182-194. https://doi.org/10.1016/j.psyneuen.2015.06.011

Stoffel, M., Neubauer, A.B. & Ditzen, B. 2021. How to assess and interpret everyday life salivary cortisol measures: a tutorial on practical and statistical considerations. *Psychoneuroendocrinology*:105391. https://doi.org/10.1016/j.psyneuen.2021.105391

Sugaya, N., Izawa, S., Ogawa, N., Shirotsuki, K. & Nomura, S. 2020. Association between hair cortisol and diurnal basal cortisol levels: a 30-day validation study. *Psychoneuroendocrinology*, 116:104650. https://doi.org/10.1016/j.psyneuen.2020.104650

Sullivan, G.M. & Feinn, R. 2012. Using effect size—or why the P value is not enough. *Journal of Graduate Medical Education*, 4(3):279-282. https://doi.org/10.4300/JGME-D-12-00156.1

Taves, M.D., Gomez-Sanchez, C.E. & Soma, K.K. 2011. Extra-adrenal glucocorticoids and mineralocorticoids: evidence for local synthesis, regulation, and function. *American Journal of Physiology-Endocrinology and Metabolism*, 301(1):E11-24. https://doi.org/10.1152/ajpendo.00100.2011

Ten, S., New, M. & Maclaren, N. 2001. Clinical review 130: Addison's disease 2001. *The Journal of Clinical Endocrinology & Metabolism*, 86(7):2909-2922. https://doi.org/10.1210/jcem.86.7.7636

Thiboutot, D., Gilliland, K., Cong, Z., Jabara, S., McAllister, J.M., Sivarajah, A. & Clawson, G. 2003. Human skin is a steroidogenic tissue: steroidogenic enzymes and cofactors are expressed in epidermis, normal sebocytes, and an immortalized sebocyte cell line (SEB-1). *Journal of Investigative Dermatology*, 120(6):905-914. https://doi.org/10.1046/j.1523-1747.2003.12244.x

Tolmay, C.M., Malan, L. & Van Rooyen, J.M. 2012. The relationship between cortisol, C-reactive protein and hypertension in African and Causcasian women: the POWIRS study: cardiovascular topics. *Cardiovascular Journal of Africa*, 23(2):78-84. https://doi.org/10.5830/cvja-2011-035

Tomlinson, J.W. & Stewart, P.M. 2001. Cortisol metabolism and the role of 11beta-hydroxysteroid dehydrogenase. *Best Practice & Reasearch Clinical Endocrinology & Metabolism*, 15(1):61-78. https://doi.org/10.1053/beem.2000.0119

Tsigos, C. & Chrousos, G.P. 2002. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *Journal of Psychosomatic Research*, 53(4):865-871. https://doi.org/10.1016/s0022-3999(02)00429-4

van den Berg, R.A., Hoefsloot, H.C., Westerhuis, J.A., Smilde, A.K. & van der Werf, M.J. 2006. Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genomics*, 7:142. https://doi.org/10.1186/1471-2164-7-142

Van Ockenburg, S., Schenk, H., van Der Veen, A., van Rossum, E., Kema, I. & Rosmalen, J. 2016. The relationship between 63 days of 24-h urinary free cortisol and hair cortisol levels in 10 healthy individuals. *Psychoneuroendocrinology*, 73:142-147. https://doi.org/10.1016/j.psyneuen.2016.07.220

Van Voorhees, E.E., Dennis, M.F., Calhoun, P.S. & Beckham, J.C. 2014. Association of DHEA, DHEAS, and cortisol with childhood trauma exposure and post-traumatic stress disorder. *International Clinical Psychopharmacology*, 29(1):56-62. https://doi.org/10.1097/YIC.0b013e328364ecd1

Van Zyl, S., Van der Merwe, L.J., Walsh, C.M., Groenewald, A.J. & Van Rooyen, F.C. 2012. Risk-factor profiles for chronic diseases of lifestyle and metabolic syndrome in an urban and

rural setting in South Africa. *African Journal of Primary Health Care and Family Medicine*, 4(1):1-10. https://doi.org/10.4102%2Fphcfm.v4i1.346

Vegiopoulos, A. & Herzig, S. 2007. Glucocorticoids, metabolism and metabolic diseases. *Molecular and Cellular Endocrinology*, 275(1-2):43-61. https://doi.org/10.1016/j.mce.2007.05.015

Venter, G., van der Berg, C.L., van der Westhuizen, F.H. & Erasmus, E. 2021. Health Status Is Affected, and Phase I/II Biotransformation Activity Altered in Young Women Using Oral Contraceptives Containing Drospirenone/Ethinyl Estradiol. *International Journal of Environmental Research and Public Health*, 18(20):10607. https://doi.org/10.3390/ijerph182010607

Voegel, C.D., Hofmann, M., Kraemer, T., Baumgartner, M.R. & Binz, T.M. 2020. Endogenous steroid hormones in hair: Investigations on different hair types, pigmentation effects and correlation to nails. *Steroids*, 154:108547. https://doi.org/10.1016/j.steroids.2019.108547

Waite, E.J., McKenna, M., Kershaw, Y., Walker, J.J., Cho, K., Piggins, H.D. & Lightman, S.L. 2012. Ultradian corticosterone secretion is maintained in the absence of circadian cues. *European Journal of Neuroscience*, 36(8):3142-3150. https://doi.org/10.1111/j.1460-9568.2012.08213.x

Wal, P., Kumar, B., Bhandari, A., Rai, A. & Wal, A. 2010. Bioanalytical method development-determination of drugs in biological fluids. *Journal of Pharmaceutical Science and Technology*, 2(10):333-347.

Walker, J.J., Spiga, F., Waite, E., Zhao, Z., Kershaw, Y., Terry, J.R. & Lightman, S.L. 2012. The origin of glucocorticoid hormone oscillations. *PLoS Biol*, 10(6):e1001341. https://doi.org/10.1371/journal.pbio.1001341

Walker, J.J., Spiga, F., Gupta, R., Zhao, Z., Lightman, S.L. & Terry, J.R. 2015. Rapid intra-adrenal feedback regulation of glucocorticoid synthesis. *Journal of the Royal Society Interface*, 12(102):20140875. https://doi.org/10.1098/rsif.2014.0875

Weckesser, L.J., Plessow, F., Pilhatsch, M., Muehlhan, M., Kirschbaum, C. & Miller, R. 2014. Do venepuncture procedures induce cortisol responses? A review, study, and synthesis for stress research. *Psychoneuroendocrinology*, 46:88-99. https://doi.org/10.1016/j.psyneuen.2014.04.012

Wester, V.L. & van Rossum, E.F. 2015. Clinical applications of cortisol measurements in hair. *European Journal of Endocrinology*, 173(4):M1-10. https://doi.org/10.1530/EJE-15-0313

Wiegratz, I., Kutschera, E., Lee, J., Moore, C., Mellinger, U., Winkler, U. & Kuhl, H. 2003a. Effect of four different oral contraceptives on various sex hormones and serum-binding globulins. *Contraception*, 67(1):25-32. https://doi.org/10.1016/S0010-7824(02)00436-5

Wiegratz, I., Kutschera, E., Lee, J.H., Moore, C., Mellinger, U., Winkler, U.H. & Kuhl, H. 2003b. Effect of four oral contraceptives on thyroid hormones, adrenal and blood pressure parameters. *Contraception*, 67(5):361-366. https://doi.org/10.1016/s0010-7824(03)00006-4

Wooding, K.M., Hankin, J.A., Johnson, C.A., Chosich, J.D., Baek, S.W., Bradford, A.P., . . . Santoro, N. 2015. Measurement of estradiol, estrone, and testosterone in postmenopausal human serum by isotope dilution liquid chromatography tandem mass spectrometry without derivatization. *Steroids*, 96:89-94. https://doi.org/10.1016/j.steroids.2015.01.007

Wosu, A.C., Valdimarsdottir, U., Shields, A.E., Williams, D.R. & Williams, M.A. 2013. Correlates of cortisol in human hair: implications for epidemiologic studies on health effects of chronic stress. *Annals of Epidemiology*, 23(12):797-811 e792. https://doi.org/10.1016/j.annepidem.2013.09.006

Wosu, A.C., Gelaye, B., Valdimarsdottir, U., Kirschbaum, C., Stalder, T., Shields, A.E. & Williams, M.A. 2015. Hair cortisol in relation to sociodemographic and lifestyle characteristics in a multiethnic US sample. *Annals of Epidemiology*, 25(2):90-95, 95 e91-92. https://doi.org/10.1016/j.annepidem.2014.11.022

Wudy, S.A., Schuler, G., Sanchez-Guijo, A. & Hartmann, M.F. 2018. The art of measuring steroids: Principles and practice of current hormonal steroid analysis. *The Journal of Steroid Biochemistry and Molecular Biology*, 179:88-103. https://doi.org/10.1016/j.jsbmb.2017.09.003

Xia, J. & Wishart, D.S. 2016. Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis. *Current Protocols in Bioinformatics*, 55(1):14 10 11-14 10 91. https://doi.org/10.1002/cpbi.11

Xue, X., Zheng, C., Jifeng, L., Jing, Z., Huihua, D. & Zuhong, L. 2013. Study on dissolution mechanism of cortisol and cortisone from hair matrix with liquid chromatography–tandem mass spectrometry. *Clinica Chimica Acta*, 421:62-72. https://doi.org/10.1016/j.cca.2013.02.035

Yeh, P.T., Kautsar, H., Kennedy, C.E. & Gaffield, M.E. 2022. Values and preferences for contraception: A global systematic review. *Contraception*, 111:3-21. https://doi.org/10.1016/j.contraception.2022.04.011

Young, A.H., Gallagher, P. & Porter, R.J. 2002. Elevation of the cortisol-dehydroepiandrosterone ratio in drug-free depressed patients. *American Journal of Psychiatry*, 159(7):1237-1239. https://doi.org/10.1176/appi.ajp.159.7.1237

Zhang, J., Li, J., Xu, Y., Yang, J., Chen, Z. & Deng, H. 2013. Characteristics of novel hair-based biomarker for the activity assessment of 11beta-hydroxysteroid dehydrogenase. *Clinica Chimica Acta*, 426:25-32. https://doi.org/10.1016/j.cca.2013.08.022

Zhang, Q., Chen, Z., Chen, S., Xu, Y. & Deng, H. 2017. Intraindividual stability of cortisol and cortisone and the ratio of cortisol to cortisone in saliva, urine and hair. *Steroids*, 118:61-67. https://doi.org/10.1016/j.steroids.2016.12.008

Zhang, Q., Chen, Z., Chen, S., Yu, T., Wang, J., Wang, W. & Deng, H. 2018. Correlations of hair level with salivary level in cortisol and cortisone. *Life Sciences*, 193:57-63. https://doi.org/10.1016/j.lfs.2017.11.037

ANNEXURE A: SUPPLEMENTARY RESULTS

Supplementary Results table of contents

SUPPLEMENTARY RESULTS

Table S 1: Source parameter values that were tested for the non-derivatised method. Parameters include capillary, nebulizer and nozzle voltages; drying gas flow and temperature; and sheath gas flow and temperature. The steps within the rages tested are indicated.

Parameter	Start Value	End Value	Step Size
Capillary voltage (V)	0	6000	500
Drying Gas Flow (L/min)	4	12	2
Drying Gas Temp (˚C)	100	350	50
Nebulizer Voltage (V)	20	60	10
Nozzle Voltage (V)	0	2000	500
Sheath Gas Flow (L/min)	6	12	2
Sheath Gas Temp (˚C)	100	400	50

Table S 2: Preliminary mobile phase gradient for non-derivatised steroids. The column temperature was 40°C and the flow rate was 0.4 ml/min.

Time (min)	%B
0	40
6	70
7	80
8	100
9	100
11	40

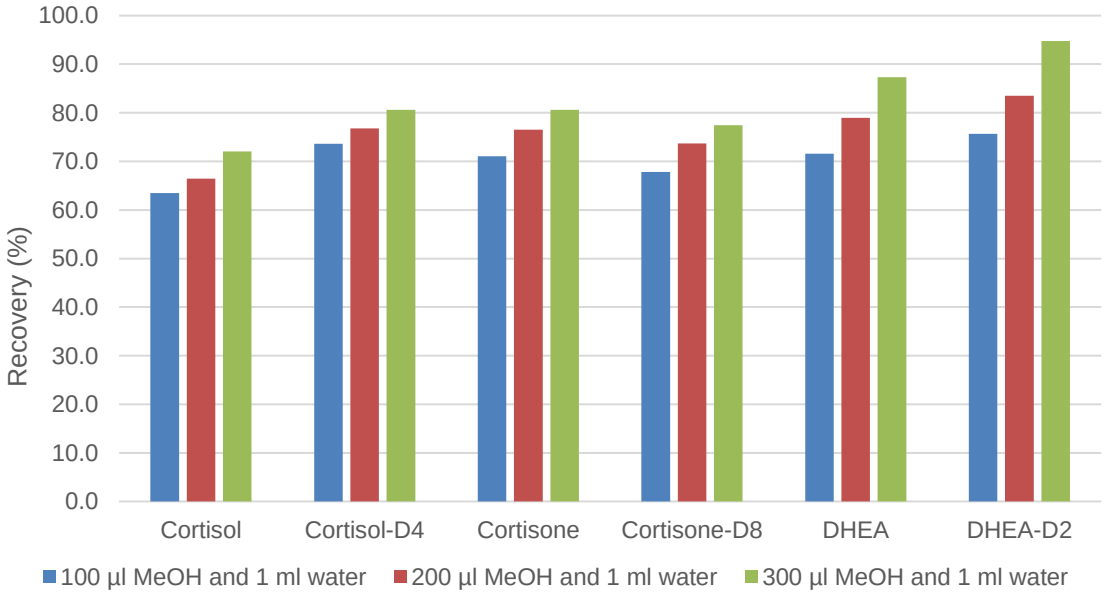


Figure S 1: Visual illustration of analyte and stable isotope recoveries in a standard mixture from different loading solutions.

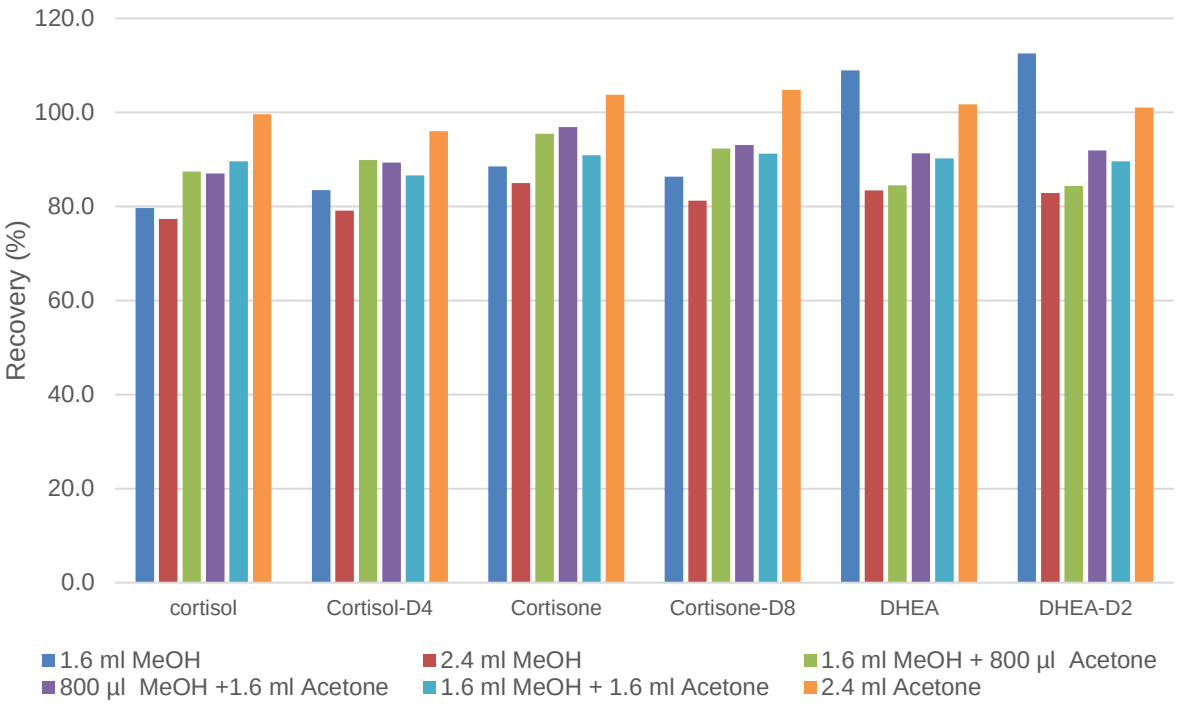


Figure S 2: Visual illustration of analyte and stable isotope recoveries in a standard mixture from different elution solvents.

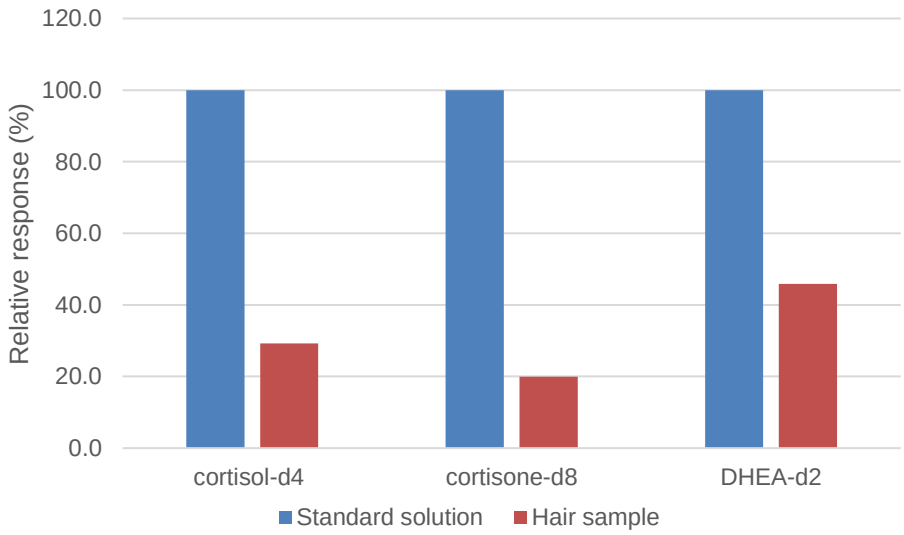


Figure S 3: Visual illustration of stable isotope responses in sample compared to responses in standard solution. Responses are expressed as a percentage of the response in standard solution.

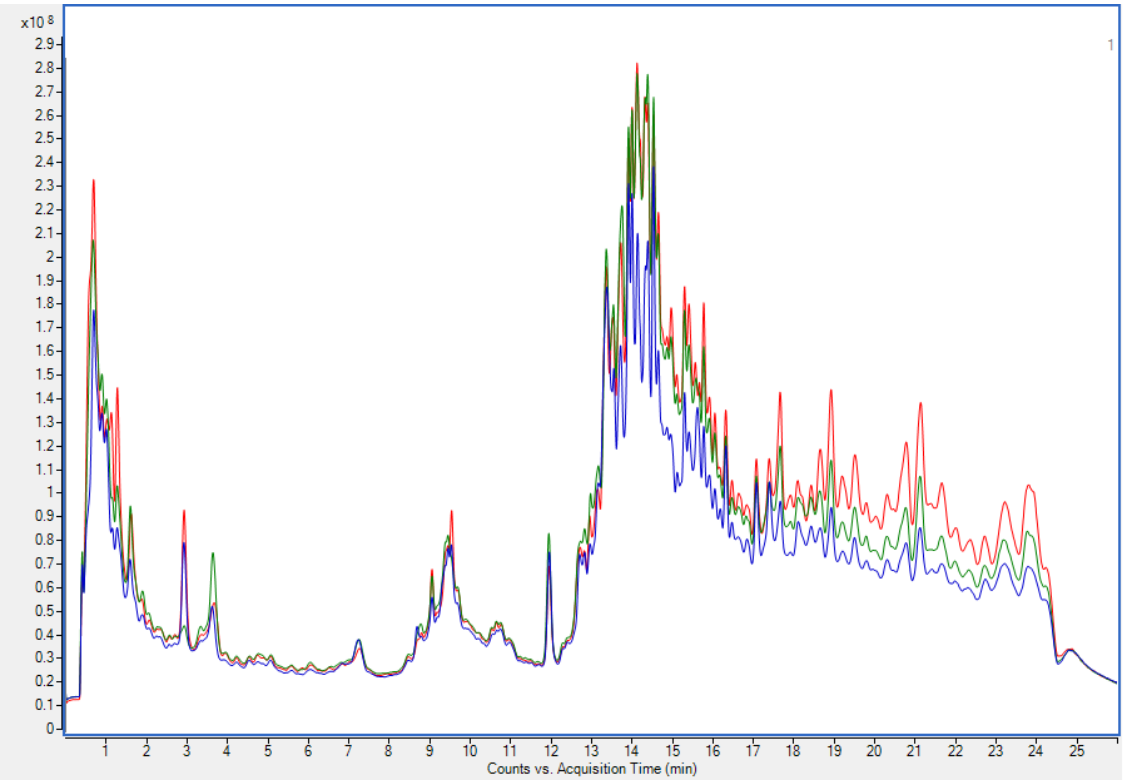


Figure S 4: Chromatogram of full scans showing the effects of wash solvent on clean-up of background using different wash solvents. The solvents tested included 100% water (red), 20% methanol (green), and 30% methanol (blue).

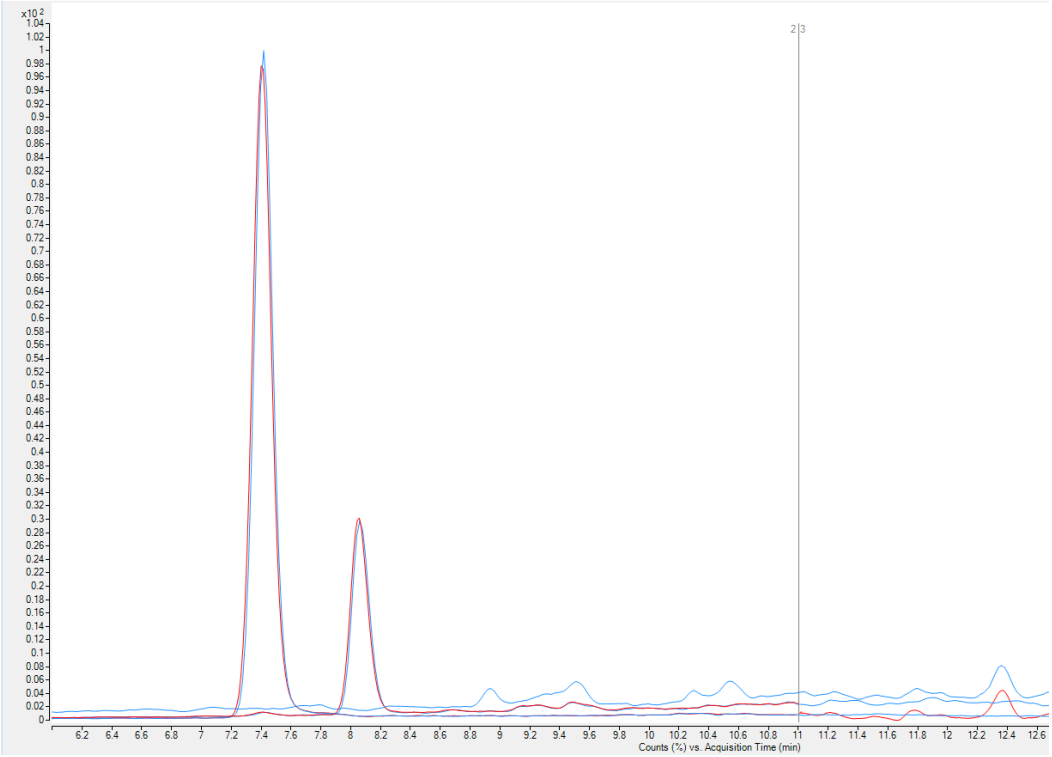


Figure S 5: MRM chromatogram showing improvement of MS signals by adding time segments that scan for fewer compounds at a time and optimising dwell times. Blue: before addition of time segments, Red: after addition of time segments.

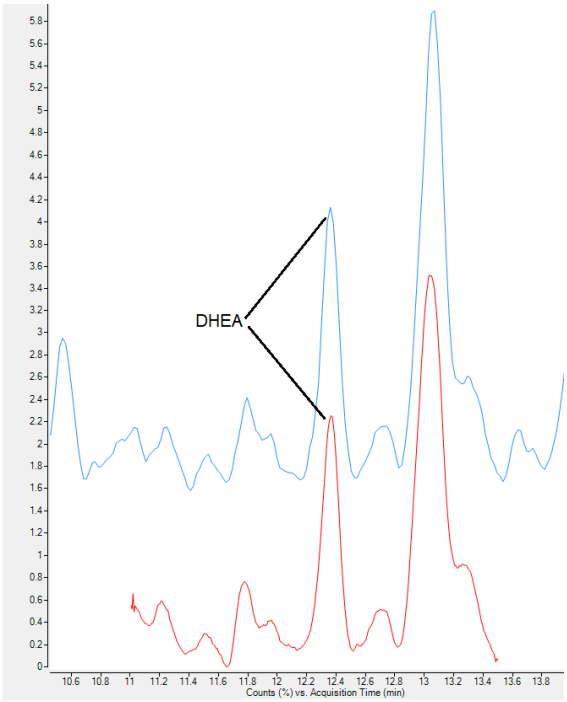


Figure S 6: MRM chromatogram showing the reduction of background for DHEA when adding a time segment that only scans for DHEA and its stable isotope. Blue: before addition of time segments, Red: after addition of time segments.

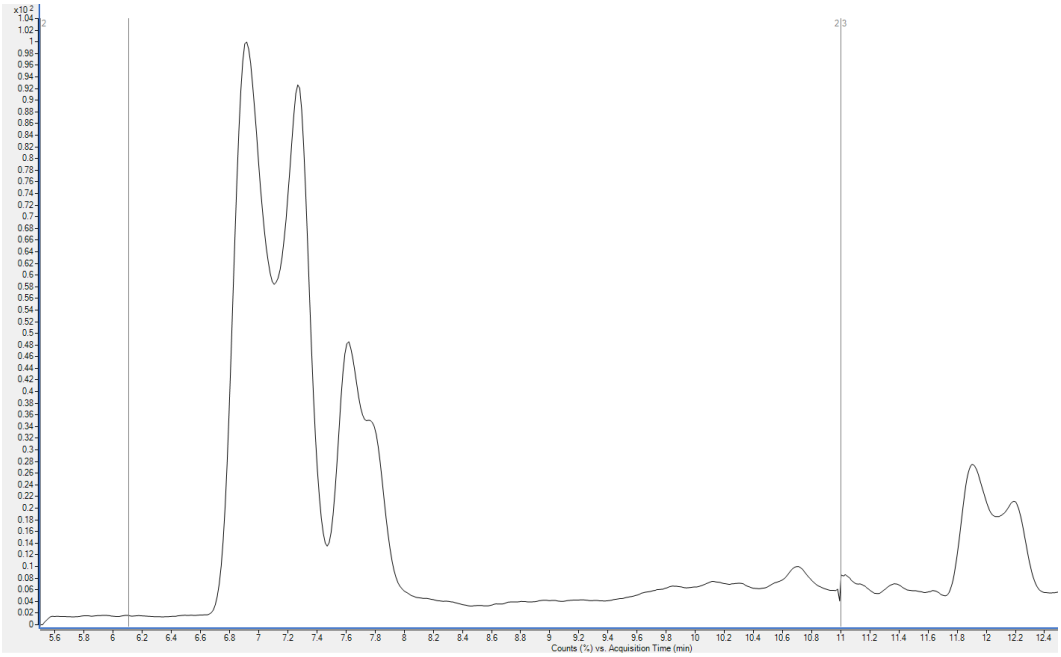
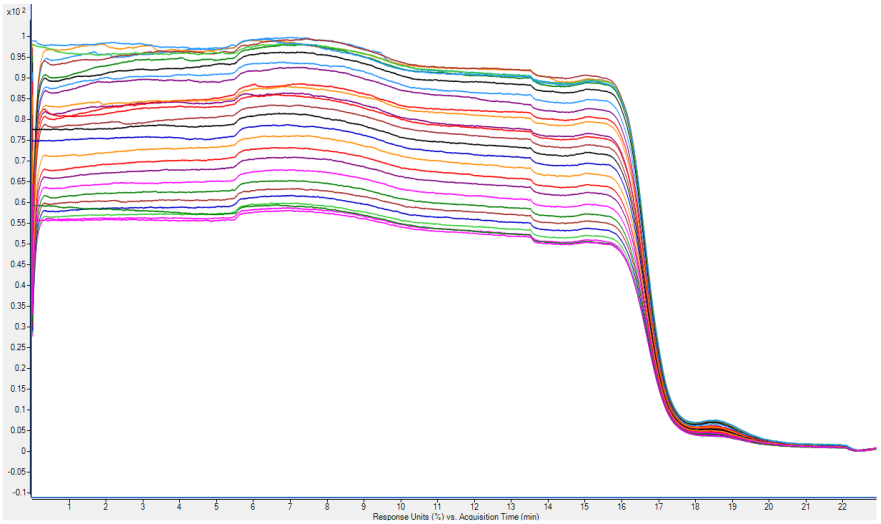


Figure S 7: Total ion chromatogram displaying split peaks due to guard column blockage.

a)



b)

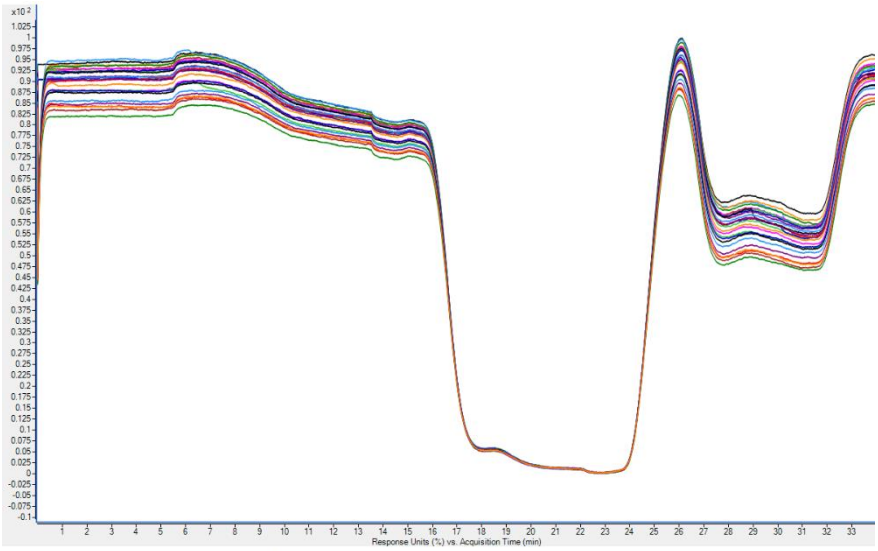


Figure S 8: Visual illustration of the increase in binary pump backpressure with each injection a) before and b) after adding a 10-minute section at 5% mobile phase B.

Table S 3: Source parameter values that were tested for the derivatised method. Parameters include capillary, nebulizer and nozzle voltages; drying gas flow and temperature; and sheath gas flow and temperature. The steps within the rages tested are indicated.

Parameter	Start Value	End Value	Step Size
Capillary voltage (V)	500	5000	500
Drying Gas Flow (L/min)	4	12	2
Drying Gas Temp (˚C)	100	300	50
Nebulizer (psi)	20	60	10
Nozzle Voltage (V)	0	2000	500
Sheath Gas Flow (L/min)	6	12	2
Sheath Gas Temp (˚C)	200	400	50

Table S 4: Retention times of preliminary separation gradient for the derivatised method.

Analyte	Retention time (min)
Cortisol-2HP	4.67
Cortisone-2HP	4.52
DHEA-2HP	5.41

Table S 5: The descriptive data of the variables investigated including the mean, median and range, as well as the expected median and range from literature. Values that were below the LLOQ were not included.

Variable	Mean	Median(range)	Expected median(range)
Cortisol (pg/mg)	3.90	4.15(0.92-34.31)	7.06(0.80-91.20)
Cortisone (pg/mg)	7.26	7.66(1.52-36.36)	25.22(6.1-542.2)
Total glucocorticoids (pg/mg)	11.28	12.07(2.87-49.49)	38.98(7.9-274.2)
Cortisol/cortisone	0.74	0.62(0.13-5.22)	0.29(0.03-10.05)

Table S 6: The descriptive data of variables in each sample group, including the range, mean, median and standard deviation. CU: Caucasian COC-users, CN: Caucasian non-users, BN: Black African non-users.

Variable	Sample group	Range	Median	Mean	Standard deviation
Cortisol (pg/mg)	CU	0.92-8.29	2.39	2.76	1.57
	CN	0.58-17.30	2.17	3.23	3.43
	BN	0.64-17.33	2.76	4.62	4.46
Cortisone (pg/mg)	CU	2.6-18.22	6.67	7.27	3.70
	CN	1.84-19.54	6.32	6.65	3.85
	BN	0.21-36.36	2.98	7.45	10.11
Total glucocorticoids (pg/mg)	CU	3.52-26.51	8.65	10.03	5.17
	CN	2.50-36.84	8.354	9.88	6.98
	BN	1.15-49.49	6.11	12.07	14.08
Cortisol/cortisone	CU	0.25-0.53	0.40	0.38	0.07
	CN	0.13-0.89	0.39	0.45	0.21
	BN	0.34-9.143	0.95	1.69	2.25